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TARGETING MTHFD1 AND MTHFD2 AS CANCER TREATMENT

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Targeting MTHFD1 and MTHFD2 as cancer treatment

Thesis for Doctoral Degree (Ph.D.)

By

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"The world is full of great and wonderful things for those who are ready for them."

— Moominpappa (Moominpappa at Sea)

Popular science summary of the thesis

Cancer is a collective term for many diseases characterized by uncontrolled cell proliferation and spreading of malignant cells into the surrounding tissues in the body. Cancer is often treated with ionizing radiation (radiotherapy) or drugs that halt the growth of cancer cells (chemotherapy). However, these therapies can cause major adverse effects in patients since radio- and chemotherapy not only target tumor but also healthy tissue. Moreover, cancer cells can become resistant to these treatments enabling the cancer to return. Therefore, there is a need to develop new, more cancer-specific therapies.

One way to combat cancer is to target tumor cell metabolism. Metabolism describes the life-sustaining building and breakdown of organic molecules in cells which are catalyzed by enzymes. Enzymes are proteins that take part in so called metabolic pathways in the cell. Cancer cells reprogram their own metabolism to support rapid proliferation and enable spreading. This means that the metabolism in cancer cells works differently compared to normal cells. One metabolic pathway that is reprogrammed in cancer cells is the one-carbon (1C) metabolism which takes place in both the cell's mitochondria and cytosol. This pathway produces 1C units that are needed for making building blocks for DNA. Since cancer cells divide rapidly, they need a lot of these building blocks to make more DNA and allow the cancer cell to divide and form new cells. Several enzymes are involved in the 1C metabolism, including MTHFD1 and MTHFD2. Because the 1C metabolism is altered in cancer cells compared to normal, non-proliferating cells, it represents a potentially cancer-specific vulnerability which could be exploited therapeutically for the development of new drugs with less side effects than conventional chemotherapy drugs.

In this thesis, we aimed to develop compounds targeting specifically the 1C pathway enzymes MTHFD1 and MTHFD2, in order to find improved, more tolerable drugs for cancer treatment. Moreover, we studied whether MTHFD2 has an additional role besides its normal function in the 1C metabolism in protecting cancer cells' DNA from damage caused by ionizing radiation.

In **Papers I** and **II**, we develop MTHFD1/2 inhibitors and uncover how these compounds kill cancer cells while largely sparing normal cells. We show that the cancer-killing effect of these compounds, which mainly target MTHFD1 in the cytosol, requires expression and activity of MTHFD2 in the mitochondria. Normal cells do not have as much of the MTHFD2 protein as cancer cells which partly explains the cancer-specificity of these inhibitors. We demonstrate that MTHFD1/2 inhibitors cause a shortage of a crucial DNA building block, called thymidylate, which leads to problems with DNA synthesis and ultimately kills cancer cells. We propose that these inhibitors could be a new promising strategy to treat cancer.

In **Paper III**, we demonstrate that MTHFD2 is needed in cancer cells to help the repair of damaged DNA and promote cancer cell survival after treatment with ionizing radiation, which causes serious damage to the genetic material. We show that MTHFD2 gets recruited to the cell's nucleus following ionizing radiation and is part of the early response to DNA damage. These findings suggest that targeting MTHFD2 may help to make cancer cells more sensitive to radiotherapy and open up exciting avenues for future research.

In summary, our work highlights the therapeutic potential of targeting the 1C metabolism enzymes MTHFD1 and MTHFD2 in cancer and contributes to the development of new, more cancer-selective treatment options.

Abstract

One-carbon (1C) metabolism provides building blocks for nucleotide synthesis and therefore plays a central role in DNA replication and repair. To sustain rapid proliferation, cancer cells often upregulate their 1C metabolism, including the enzymes MTHFD1 and MTHFD2, as a part of their metabolic rewiring. Previously, MTHFD2 in particular has been indicated as a potential drug target, mainly due to its cancer-enriched expression profile. Interestingly, both MTHFD1 and MTHFD2 have also emerging nuclear functions besides their canonical metabolic activities in the 1C pathway. However, the nuclear localization of MTHFD2 and its role in the DNA damage response are not well understood. Moreover, evaluation of the therapeutic potential of targeting MTHFD1 and MTHFD2 in cancer is hampered by the lack of potent inhibitors of these enzymes. In this thesis, we aimed to develop small-molecule MTHFD1/2 inhibitors and characterize their mechanism of action, as well as study the nuclear role of MTHFD2 in DNA repair.

In **Paper I**, we develop a series of small-molecule MTHFD1/2 inhibitors, including TH9619. We study the mechanism of action of these inhibitors and show that they cause thymidylate depletion, followed by excessive misincorporation of uracil into DNA, induction of replication stress and cell death in acute myeloid leukemia cells. These new inhibitors selectively induced apoptosis in leukemia cells while largely sparing non-tumorigenic cells and displayed efficacy in a mouse xenograft model of acute myeloid leukemia.

In **Paper II**, we further investigate the mechanism of action of MTHFD1/2 inhibitors, focusing on TH9619. We reveal that TH9619 engages with nuclear MTHFD2 but does not disrupt formate overflow from mitochondria since it cannot target mitochondrial MTHFD2. Mechanistically, TH9619 caused accumulation of 10-formyl-tetrahydrofolate downstream of mitochondrial formate release due to its inhibition of MTHFD1. Trapping of 10-formyl-tetrahydrofolate ultimately led to thymidylate depletion and cell death in MTHFD2-expressing colorectal cancer cells.

Lastly, in **Paper III**, we identify a nuclear role of MTHFD2 in the early steps of DNA double-strand break repair in cancer cells. We found that MTHFD2 rapidly accumulated in the nucleus following ionizing radiation, which was mediated by the ATM and DNA-PK kinases, and co-localized with DNA damage sites. Depletion of MTHFD2 led to impaired phosphorylation of BRCA1, defective DNA end resection and decreased HR and NHEJ repair activity. Moreover, inhibition of MTHFD2 with TH9619 exacerbated DNA damage after irradiation in repair-proficient cancer cells and synergized with PARP inhibitors.

In conclusion, this thesis details the complex mechanism of action of MTHFD1/2 inhibitors and highlights their therapeutic potential in cancer. Our work also demonstrates a critical role of MTHFD2 in facilitating double-strand break repair.

List of scientific papers

Scientific papers included in the thesis:

I. Bonagas, N.*, Gustafsson, N. M. S.*, Henriksson, M.*, **Marttila, P.***, Gustafsson, R., Wiita, E., Borhade, S., Green, A. C., Vallin, K., Sarno, A., Svensson, R., Göktürk, C., Pham, T., Jemth, A-S., Loseva, O., Cookson, V., Kiweler, N., Sandberg, L., Rasti, A., Unterlass, J. E., Haraldsson, M., Andersson, Y., Scaletti, E. R., Bengtsson, C., Paulin, C. B. J., Sanjiv, K., Abdurakhmanov, E., Pudelko, L., Kunz, B., Desroses, Iliev, P., Färnegårdh, K., Krämer, A., Garg, N., Michel, M., Häggblad Sahlberg, S., Jarvius, M., Kalderén, C., Bögedahl Jensen, A., Almlöf, I., Karsten, S., Zhang, S. M., Häggblad, M., Eriksson. A., Liu, J., Glinghammar, B., Nekhotiaeva, N., Klingegård, F., Koolmeister, T., Martens, U., Llona-Minguez, S., Moulson, R., Nordström, H., Parrow, V., Dahllund, L., Sjöberg, B., Vargas, I. L., Vo, D. D., Wannberg, J., Knapp, S., Krokan, H. E., Arvidsson, P. I., Scobie, M., Meiser, J., Stenmark, P., Warpman Berglund, U., Homan, E. J., Helleday, T. **Pharmacological targeting of MTHFD2 suppresses acute myeloid leukemia by inducing thymidine depletion and replication stress.** *Nature Cancer* 3, 156-172 (2022)

II. Green, A. C.*, **Marttila, P.***, Kiweler, N.*, Chalkiadaki, C., Wiita, E., Cookson, V., Lesur, A., Eiden, K., Bernardin, F., Vallin, K.S.A., Borhade, S., Long, M., Kamali Ghahe, E., Jiménez-Alonso, J.J., Jemth, A-S., Loseva, O., Mortusewicz, O., Meyers, M., Viry, E., Johansson, A.I., Hodek, O., Homan, E., Bonagas, N., Ramos, L., Sandberg, L., Frödin, M., Moussay, E., Slipicevic, A., Letellier, E., Paggetti, J., Storgaard Sørensen, C., Helleday, T.#, Henriksson, M.#, Meiser, J.# **Formate overflow drives toxic folate trapping in MTHFD1 inhibited cancer cells.** *Nature Metabolism* 5, 642-659 (2023)

III. **Marttila, P.***, Bonagas, N.*, Chalkiadaki, C., Schelzig, K., Farhat, C.M., Hondema, A., Albers, J., Wiita, E., Rasti, A., Warpman Berglund, U., Slipicevic, A., Mortusewicz, O., Helleday, T. **Targeting MTHFD2 impairs homologous recombination and sensitizes cancer cells to PARP inhibitors.** *Manuscript submitted*

* These authors contributed equally to this work
# These authors jointly supervised this work

Scientific papers not included in the thesis:

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V. Škerlová, J., Unterlass, J., Göttman, M., **Marttila, P.,** Homan, E., Helleday, T., Jemth, A-S., Stenmark, P. **Crystal structures of human PAICS reveal substrate and product binding of an emerging cancer target.** *Journal of Biological Chemistry* 295, 11656-11668 (2020)

VI. Karsten, S., Fiskesund, R., Zhang, X.-M., **Marttila, P.,** Sanjiv, K., Pham, T., Rasti, A., Bräutigam, L., Almlöf, I., Marcusson-Ståhl, M., Sandman C., Platzack, B., Harris, R. A., Kalderén, C., Cederbrant, K., Helleday, T., Warpman Berglund, U. **MTH1 as a target to alleviate T cell driven diseases by selective suppression of activated T cells.** *Cell Death & Differentiation* 29, 246-261 (2021)

VII. Michel, M.*, Benítez-Buelga, C.*, Calvo, P.A.†, Hanna, B.M.F.†, Mortusewicz, O.†, Masuyer, G.†, Davies, J.†, Calvete, O.†, Wallner, O.†, Sanjiv, K.†, Albers, J.J., Castañeda-Zegarra, S., Jemth, A.S., Visnes, T., Sastre-Perona, A., Danda, A.N., Homan, E.J., Marimuthu, K., Zhenjun, Z., Chi, C.N., Sarno, A., Wiita, E., Von Nicolai, C., Komor, A.J., Rajagopal, V., Müller, S., Hank, E.C., Varga, M., Scaletti, E.R., Pandey, M., Karsten, S., Haslene-Hox, H., Loevenich, S., **Marttila, P.**, Rasti, A., Mamonov, K., Ortis, F., Schömberg, F., Loseva, O., Stewart, J., D'Arcy-Evans, N., Koolmeister, T., Henriksson, M., Michel, D., de Ory, A., Acero, L., Calvete, O., Scobie, M., Hertweck, C., Vilotijevic, I., Kalderén, C., Osorio, A., Perona, R., Stolz, A., Stenmark, P., Warpman Berglund, U., De Vega, M., Helleday, T. **Small-molecule activation of OGG1 increases oxidative DNA damage repair by gaining a new function.** *Science* 376, 1471-1476 (2022)

VIII. Largeot, A., Klapp, V., Viry, E., Gonder, S., Fernandez Botana, I., Blomme, A., Benzarti, M., Pierson, S., Duculty, C., **Marttila, P.,** Wierz, M., Gargiulo, E., Pagano, G., Perez Hernandez, D., Chakraborty, S., Ysebaert, L., François, J-H., Cortez, S., Berchem, G., Efremov, D.G., Dittmar, G., Szpakowska, M., Chevigne, A., Nazarov, P.V., Helleday, T., Close, P., Meiser, J., Stamatopoulos, B., Desaubry, L., Paggetti, J., Moussay, E. **Inhibition of MYC translation through targeting of the newly identified PHB-eIF4F complex as therapeutic strategy in CLL.** *Blood* 141, 3166–3183 (2023)

IX. Zhang, S.M., Paulin, C.B.J., Shu, H., Yagüe-Capilla, M., Michel, M., **Marttila, P.,** Ortis, F., Bwanika, H.C., Dirks, C., Papagudi Vekatram, R., Wiita, E., Jemth, A-S., Almlöf, I., Loseva, O., Hormann, F.M., Koolmeister, T., Linde, E., Lee, S., Llona-Minguez, S., Haraldsson, M., Strömberg, K., Homan, E.J., Scobie, M., Lundbäck, T., Helleday, T., Rudd, S.G. **Identification and evaluation of small-molecule inhibitors against the dNTPase SAMHD1 via a comprehensive screening funnel.** *Manuscript submitted. Preprint at bioRxiv: https://doi.org/10.1101/2023.01.17.524275*

*/† These authors contributed equally to this work

Contents

List of abbreviations

AICAr	5-amino-4-imidazolecarboxamide ribonucleoside
AICAR	5-amonoimidazole-4-carboxamide ribonucleotide
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AMP	Adenine monophosphate
ATIC	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
ATRIP	ATR-interacting protein
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BrdU	5-bromo-2'-deoxyuridine
CDK	Cyclin-dependent kinase
CETSA	Cellular thermal shift assay
CHK	Checkpoint kinase
CtIP	CtBP-interacting protein
DARTS	Drug affinity responsive target stability
DC	Dehydrogenase/cyclohydrolase
DDR	DNA damage response
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dNDP	Deoxyribonucleoside diphoshate
dNMP	Deoxyribonucleoside monophoshate
dNTP	Deoxyribonucleoside triphosphate
DSB	DNA double-strand break
dsDNA	Double-stranded DNA
dTMP	Deoxythymidine monophosphate, thymidylate

dTTP	Deoxythymidine triphosphate
DTYMK	Deoxythymidylate kinase
dUDP	Deoxyuridine diphosphate
dUMP	Deoxyuridine monophosphate
dUTP	Deoxyuridine triphosphate
dUTPase	dUTP diphosphatase
EdU	5-ethyl-2′-deoxyuridine
EXO1	Exonuclease 1
FPGS	Folylpolyglutamate synthase
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HR	Homologous recombination
IMP	Inosine monophosphate
iPOND	Isolation of proteins on nascent DNA
IR	Ionizing radiation
LIG4	DNA ligase IV
MDC1	Mediator of DNA damage checkpoint protein 1
MRN	MRE11-RAD50-NBS1 complex
MTHFD1	Methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1
MTHFD1L	Methylenetetrahydrofolate dehydrogenase 1 like
MTHFD2	Bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase, mitochondrial
MTHFD2L	Methylenetetrahydrofolate dehydrogenase 2 like
MTR	Methionine synthase
NDPK	Nucleoside diphosphate kinase
NHEJ	Non-homologous end joining
PARP	Poly (ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
PCFT	Proton-coupled folate transporter

PIKK	Phosphatidylinositol 3-kinase-related kinase
PPAT	Phosphoribosyl pyrophosphate amidotransferase
PRPP	Pentose phosphate pathway
PRPP	Phosphoribosyl pyrophosphate
RAD51	RAD51 recombinase
RFC	Reduced folate transporter
RNR	Ribonucleotide reductase
RPA	Replication protein A
RS	Replication stress
SDSA	Synthesis-dependent strand-annealing
SHMT	Serine hydroxymethyl transferase
siRNA	Small interfering RNA
SSB	DNA single-strand break
ssDNA	Single-stranded DNA
THF	Tetrahydrofolate
TK	Thymidine kinase
TYMS	Thymidylate synthase
UV	Ultraviolet
XLF	XRCC4-like factor
XRCC4	X-ray repair cross-complementing protein 4
1C	One-carbon
5-CH_3-THF	5-methyl-tehtrahyrofolate
5-FU	5-fluorouracil
5,10-CH_2-THF	5,10-methylene tetrahydrofolate
10-CHO-THF	10-formyl-tetrahydrofolate
γH2AX	Histone H2AX phosphorylated at serine 139

1 Introduction

Cancer is the leading cause of premature death across the world, accounting for nearly 10 million deaths in 2020[1,2]. It is an umbrella term that comprises a vast number of different diseases characterized by abnormal cells harboring genetic mutations that enable uncontrolled proliferation and invasiveness. Cancers can originate from nearly any tissue in the body. The early 20th century marked the start of modern cancer research, accompanied by the development of radio- and chemotherapies that induce DNA damage to kill cancer cells[3,4]. For nearly a century, radio- and chemotherapy, as well as surgery have formed the cornerstones of available cancer therapies, and even today these treatments continue to be effective in curing some tumors. However, radiation and conventional chemotherapy often give rise to dose-limiting toxicities in healthy tissues, moreover, cancer cells can develop resistance to these therapies[4–6]. Targeted therapies represent a new generation of anti-cancer treatment that focus on interfering with cancer-specific molecular changes[7,8]. There is currently a great interest in understanding cancer-specific vulnerabilities, such as rewired metabolism and genome instability, and identifying novel, druggable proteins that could be exploited therapeutically as anticancer drug targets.

In this thesis work, we sought to develop and validate new inhibitors targeting cancer cell metabolism, focusing specifically on MTHFD1 and MTHFD2 which are central enzymes of the one-carbon folate metabolism. Moreover, we studied the potential role of MTHFD2 in DNA repair.

1.1 Altered metabolism as a hallmark of cancer

Metabolic reprogramming is a well-established hallmark of cancer[9,10]. Rewiring of cellular metabolism manifests as altered nutrient utilization, which enables cancer cells to shunt building blocks and energy supplies to biosynthetic pathways to support tumor growth and metastasis[11]. The metabolic needs of cancer cells also evolve as cancer progresses to secure tumor cell fitness in a changing environment[12]. Moreover, recent findings highlight that human cancers are metabolically heterogeneous, meaning that metabolic changes and vulnerabilities are not necessarily uniform across all cancers[13].

Historically, the 1920s marked the beginning of cancer metabolism research when Otto Warburg's and his team to discovered the "Warburg effect" revealing that many tumors increase their uptake of glucose and prefer to utilize glycolysis and lactose fermentation even under aerobic conditions[14,15]. For many years, Warburg and others misinterpreted these findings to indicate that mitochondria were damaged in cancer, which in turn inhibited the oxidation of glucose to CO_2 through mitochondrial respiration[16,17]. However, today we understand that this is not the root of aerobic glycolysis in cancer. In fact, mitochondria are often intact in many tumors and are capable of performing oxidative

phosphorylation. Importantly, mitochondrial metabolism is rewired in cancer to support the synthesis of anabolic precursors for macromolecular synthesis[11,18]. Nowadays, mitochondrial metabolic pathways, including one-carbon metabolism, glutaminolysis, fatty acid oxidation and redox metabolism, are considered necessary for cancer cell survival[19]. Although our understanding of the Warburg effect is still incomplete, the increased reliance of cancer cells on glycolysis has been suggested to enable redirecting of glycolysis intermediates to anabolic pathways to support proliferation. For instance, cancer cells shunt glycolytic carbon units into the pentose phosphate pathway (PPP) to generate substrates for nucleotide synthesis[11,18,20].

Another landmark event in the field of cancer metabolism took place 30 years after Warburg's discoveries when Sidney Farber reported the use of anti-folates to treat childhood acute lymphoblastic leukemia (ALL)[21]. The observations of Farber and his co-workers laid the foundations for cancer chemotherapy and highlighted folate antagonists as therapeutic agents that could inhibit nucleotide biosynthesis[22].

Despite the initial work of Warburg and Farber, the identification of tumor suppressors and oncogenes as drivers of neoplastic transformation in the 1980s led to a reduced interest in targeting altered metabolism for cancer treatment[23]. As scientific efforts were directed to developing drugs targeting oncogenic kinases[24–26], research in cancer metabolic reprogramming was overlooked. However, as the intricate interplay between metabolic reprogramming and cancer genetic alterations became clear, the interest in studying cancer metabolism was revived[20,27–29].

Today, it is appreciated that metabolic reprogramming in cancers is in part driven by genetic or epigenetic alterations in oncogenes or tumor suppressors, such as KRAS, MYC, AKT, PI3K, p53 and Rb[28,30,31]. The discovery of cancer-associated mutations in some genes coding for metabolic enzymes, as well as the accumulation of oncometabolites due to these mutations, further strengthens the direct connection between altered cancer metabolism and tumorigenesis[32–34]. A prime example is the gain-of-function mutation in the enzyme isocitrate dehydrogenase (IDH) involved in the tricarboxylic acid cycle (TCA)[35–38]. As a result, IDH gains a novel reductive activity to convert α-ketoglutarate (α-KG) to the oncometabolite 2-hydroxyglutarate (2-HG), which in turn impairs histone demethylation and subsequently blocks cell differentiation[39–41]. Consequently, inhibiting the mutant IDH would provide a way to specifically target IDH-mutant cancers. Indeed, both enasidenib and ivosidenib represent first-in-class small molecule inhibitors targeting mutant IDH enzymes, and were recently approved for the treatment of acute myeloid leukemia (AML)[42,43]. This success story encourages further research in cancer metabolism to identify other novel therapeutic targets.

Recent technological advances, like chromatography-coupled mass spectrometry, stable isotope tracers for metabolic pathway analysis, seahorse, genome editing and

integrated multi-omics approaches, have also increased the interest in studying cancer-specific metabolic alterations, as they provide more elaborate means to address metabolism-related research questions[44]. Metabolomic analyses hold great potential in terms of discovering new anticancer targets. For instance, the combination of metabolite profiling using mass spectrometry and microarray gene expression data was previously employed to uncover the expression of the mitochondrial one-carbon (1C) enzymes SHMT2, MTHFD2 and MTHFD1L to correlate with rapid cancer proliferation[45]. Importantly, analysis of microarray datasets provides valuable information on reprogramming of metabolic enzymes in cancer on a genome-wide scale[46,47].

In conclusion, metabolic reprogramming is a distinct characteristic of cancers, comprising metabolic adaptations that evolve throughout tumor progression to support cancer cell survival. Cancer cells effectively rewire their metabolism to redirect metabolic intermediates to support lipid, protein and nucleotide biosynthesis. Moreover, cancer cells also often switch to use mitochondrial enzymes for the synthesis of anabolic precursors[11,18]. Recently, there has been a re-surge of interest in studying metabolic changes associated with cancer[48]. Despite the challenges in targeting altered cancer metabolism arising from metabolic flexibility, as well as intra- and inter-tumor heterogeneity of cancers[49,50], there is potential for uncovering cancer-specific metabolic enzymes and designing rational therapies for effective cancer treatment.

1.2 DNA metabolism in cancer

1.2.1 An overview of the nucleotide synthesis pathways

Synthesis of nucleotides occurs either via the *de novo* or the salvage pathways. For the stepwise assembly of pyrimidine and purine, the *de novo* pathways utilize nitrogen and carbon atoms derived from amino acids (e.g., glutamine, aspartate, glycine and serine), glucose or bicarbonate. The salvage pathways, in turn, recycle nucleosides and nucleobases arising from either intracellular degradation of nucleic acids or extracellular uptake from the blood[51]. Both the *de novo* and salvage pathways require phosphoribosyl pyrophosphate (PRPP) as the sugar moiety, which is the activated form of ribose 5-phosphate (R5P) produced by the pentose phosphate pathway (PPP).

The ribonucleoside monophosphates (rNMPs) arising from *de novo* purine and pyrimidine pathways (IMP and UMP, respectively) are first converted to ribonucleoside diphosphates (rNDPs), which in turn are reduced to deoxyribonucleoside diphosphates (dNDPs) by the enzyme ribonucleotide reductase (RNR)[52,53]. This conversion transforms RNA building blocks to DNA building blocks (Figure 1). Thereafter, dNDPs are converted to deoxyribonucleoside triphosphates (dNTPs) by the activity of nucleoside diphosphate kinase (NDPK)[54]. Ultimately, dNTPs serve as substrates for DNA polymerases during DNA replication and repair. The intracellular levels of dNTPs are kept

low, i.e., in the micromolar range, as opposed to more abundant rNTPs. The pools of dNTPs rise in a cell cycle-dependent fashion, increasing especially in late G1 and during S phase[55,56].

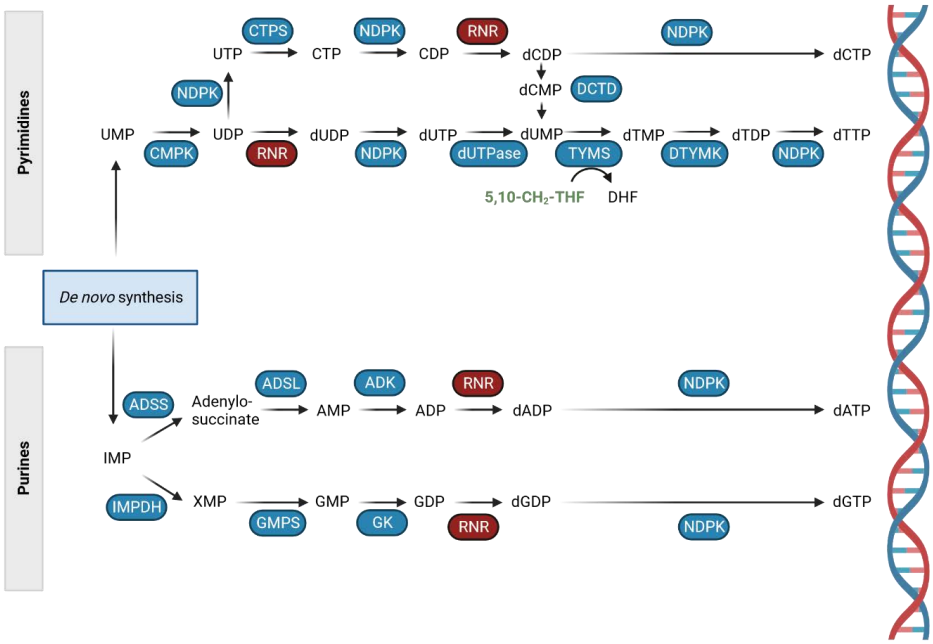

Figure 1. Simplified schematic of *de novo* dNTP synthesis in mammals. Ribonucleotide reductase (RNR), which converts ribonucleoside diphosphates to deoxyribonucleoside diphosphates is highlighted in dark red. Co-factor 5,10-methylene-tetrahydrofolate (5,10-CH_2-THF) derived from the one-carbon folate metabolism is highlighted in green. UMP, uridine monophosphate; CMPK, cytidine monophosphate kinase; NDPK, nucleoside diphosphate kinase; CTPS, CTP synthase; DCTC, deoxycytidylate deaminase; dUTPase, dUTP diphosphatase; TYMS; thymidylate synthase; DTYMK, deoxythymidylate kinase; IMP, inosine monophosphate; ADSS, adenylosuccinate synthase; IMPDH, inosine monophosphate dehydrogenase; ADSL, adenylosuccinate lyase; GMPS, guanine monophosphate synthase; ADK, adenylate kinase; GK, guanylate kinase; DHF, dihydrofolate. Image created with BioRender.com.

Enzymes involved in the purine and pyrimidine salvage pathways (Figure 2) are mainly localized to the cytoplasm, except for thymidine kinase 2 (TK2) which is expressed in the mitochondria[54]. Adenine phosphoribosyl transferase (APRT) and hypoxanthine-guanine phosphoribosyl transferase (HPRT) are involved in purine salvage and catalyze the conversion of adenine and guanine to adenosine monophosphate (AMP) and guanosine monophosphate (GMP), respectively. In addition, HPRT can generate inosine monophosphate (IMP) from hypoxanthine[54,57]. In the pyrimidine salvage pathway, uridine-cytidine kinase (UCK), deoxycytidine kinase (dCK), as well as thymidine kinases 1 and 2 (TK1, TK2), convert free pyrimidine bases to their corresponding rNMPs or deoxyribonucleoside monophosphates (dNMPs)[54,58].

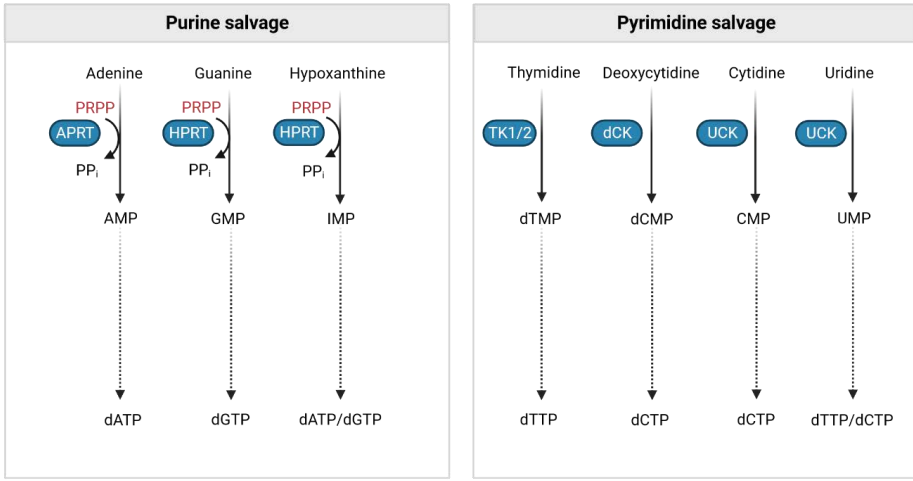

Figure 2. Simplified overview of purine and pyrimidine salvage pathways. Phosphoribosyl pyrophosphate (PRPP) is derived from the pentose-phosphate pathway and is highlighted in red. Dashed arrows represent multiple enzymatic steps. APRT, Adenine phosphoribosyl transferase; HPRT, hypoxanthine-guanine phosphoribosyl transferase; TK, thymidine kinase; dCK, deoxycytidine kinase; UCK; PPi, pyrophosphate. Image created with BioRender.com.

The *de novo* pathways are highly energy-demanding, while the salvage pathways consume less energy. Generally, the salvage pathways represent the main sources of purines and pyrimidines under normal physiological conditions and for resting or differentiated cells. In cancer cells, however, due to their increased demand for nucleic acids, salvaging of nucleotides is often insufficient to sustain rapid proliferation. Therefore, cancer cells frequently rely more on the *de novo* pathways to secure a continuous supply of nucleotides[59–61]. To supply substrates needed for the synthesis of nucleotide precursors, cancer cells also upregulate their 1C metabolism[62].

1.2.2 One-carbon folate metabolism

One-carbon (1C) metabolism encompasses two coupled pathways, namely the folate and methionine cycles (Figure 3), and is instrumental for the cellular production of nucleic acids, lipids, proteins and redox co-factors. Folate-mediated 1C metabolism is one of the most deregulated metabolic pathways in cancer and of high clinical relevance[45,47,62,63]. The 1C metabolism has for decades been successfully (but not specifically) targeted for treatment of various human hematological and solid cancers with drugs such as methotrexate and pemetrexed[64–66]. One-carbon folate metabolism is intricately linked to nucleotide biosynthesis, and its activity is required to transfer 1C units to both *de novo* thymidylate and purine synthesis pathways[60] (Figure 3). Consequently, this pathway plays an integral role in genome maintenance through the regulation of intracellular nucleotide pools[67]. Moreover, folate metabolism supports DNA and protein methylation by shuttling 1C units to the methionine cycle (Figure 3).

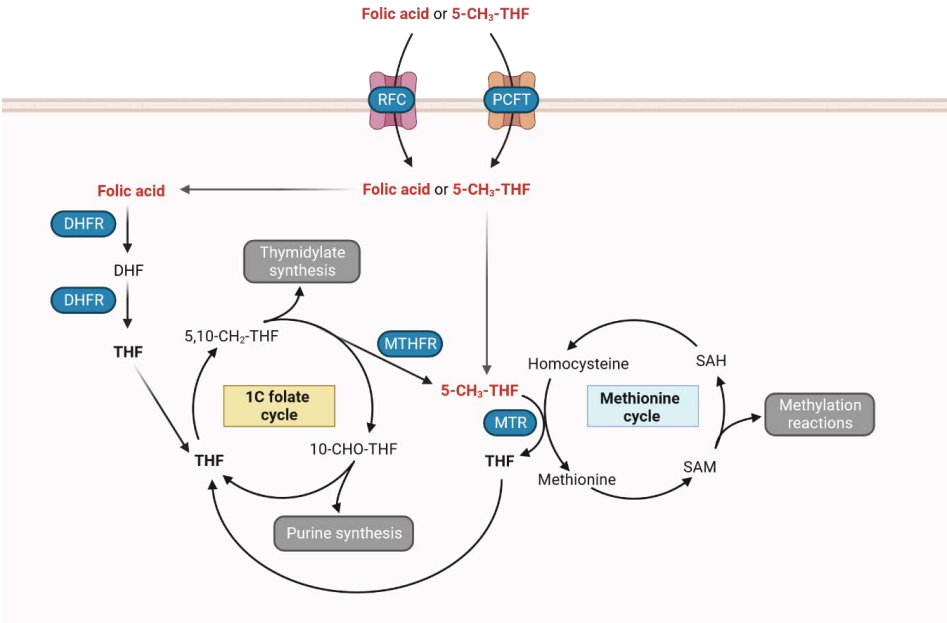

Figure 3. Simplified overview of the 1C metabolism consisting of the folate and methionine cycles. 5-methyl-tetrahydrofolate (5-CH_3-THF) or folic acid serve as the sources of free THF and are highlighted in red. They are transported into the cell by the reduced folate transporter (RFC or SLC19A1) or the proton-coupled folate transporter (PCFT or SLC46A1). Inside the cell, the enzyme methionine synthase (MTR) converts 5-CH_3-THF into free THF and methionine, which then enter the folate or methionine cycle, respectively. Alternatively, reduction of folic acid by dihydrofolate reductase (DHFR) provides THF for the folate cycle. Major outputs of the folate and methionine cycles are highlighted in grey. The 1C folate cycle yields 5,10-methylene-THF (5,10-CH_2-THF) and 10-formyl-THF (10-CHO-THF) which support *de novo* thymidylate and purine synthesis, respectively. The methionine cycle yields *S*-adenosylmethionine (SAM) which acts as methyl group donor during methylation reactions catalyzed by the family of SAM-dependent methyltransferases. When methylating either DNA, RNA, lipids or proteins, methyltransferases produce S-adenosylhomocysteine (SAH) as a by-product. Between the folate and methionine cycles, methylenetetrahydrofolate reductase (MTHFR) can convert 5,10-CH_2-THF into 5-CH3-THF. Image created with BioRender.com.

The folate cycle centers around tetrahydrofolate (THF) which acts as a carrier molecule for 1C moieties throughout the folate cycle. Animals, unlike most bacteria, yeast and plants, cannot synthesize folate *de novo* and thus rely on the uptake of exogenous precursors[62]. Nearly all natural folate species absorbed from the diet are converted to 5-methyl THF (5-CH_3-THF) in the gut and liver, followed by distribution to tissue folate pools. The reduced 5-CH_3-THF is the predominant circulating folate precursor in plasma[62,68–70]. To convert 5-CH_3-THF to the biologically active THF, cells utilize the cobalamin-dependent enzyme methionine synthase (MTR), which transfers the methyl group from 5-CH_3-THF to homocysteine, yielding methionine and THF[71–73]. Thus, MTR

acts as a link between the folate and methionine cycles (Figure 3). In contrast, the conversion of folic acid (vitamin B9), which is a common synthetic food additive, requires the activity of dihydrofolate reductase (DFHR) that first catalyzes the reduction of folic acid to dihydrofolate (DHF) and then to THF[62] (Figure 3). DHFR is also the main target of the antifolate chemotherapy drug methotrexate. Uptake of folate precursors into the cell requires active transport across the cell membrane, which is carried out mainly by the anionic reduced folate transporter (RFC, SLC19A1) or the proton-coupled folate transporter (PCFT, SLC46A1)[74] (Figure 3). Another folate transporter, SLC25A32, controls the entry of cytosolic THF into the mitochondria (Figure 4)[75,76]. Glutamate tails are added to folates upon entering the cell in a process called polyglutamation, catalyzed by folylpolyglutamate synthase (FPGS). This leads to an enhanced intracellular retention of the folates due to their increased affinity to 1C folate enzymes and decreased affinity for folate export proteins[77,78]. In addition to natural folates, antifolate drugs, such as methotrexate, also undergo polyglutamation[79].

The 1C moieties carried by THF are sourced from serine, glycine and the choline degradation products dimethylglycine and methylglycine[62,67]. Of these, serine represents the major 1C donor[80]. Serine hydroxymethyl transferase enzymes (SHMT1 and SHMT2) catalyze the transfer of 1C units from serine to yield 5,10-methylene-THF (5,10-CH_2-THF). THF-bound 1C moieties can be interconverted between different oxidation states, each of which is directed to distinct biosynthetic pathways. The species to support thymidylate (dTMP) and methionine synthesis is 5,10-CH_2-THF, while 10-formyl-THF (10-CHO-THF) is shunted to *de novo* purine synthesis[62] (Figure 4).

Oxidation of 5,10-CH_2-THF requires the activity of enzymes from the methylenetetrahydrofolate dehydrogenase cyclohydrolase (MTHFD) family. In eukaryotes, the folate-mediated 1C metabolism is compartmentalized between mitochondria, cytosol and nucleus[81–83] (Figure 4). The mitochondrial pathway converts 5,10-CH_2-THF derived from serine to formate, which can enter the cytosol, and is comprised of SHMT2, the bifunctional and homodimeric methylenetetrahydrofolate dehydrogenase/cyclohydrolase (MTHFD2), or its isoform methylenetetrahydrofolate dehydrogenase 2 like (MTHFD2L), and the monofunctional methylenetetrahydrofolate dehydrogenase 1 like protein (MTHFD1L). The enzymes involved particularly in the mitochondrial folate pathway are consistently overexpressed in human tumors[45,47,84]. In the cytosolic branch of the 1C folate pathway, interconversion between 5,10-CH_2-THF and formate is performed by the trifunctional methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1 (MTHFD1). SHMT1 , in turn, is the cytosolic counterpart of SHMT2 and couples 1C units derived from serine to THF[62,67] (Figure 4).

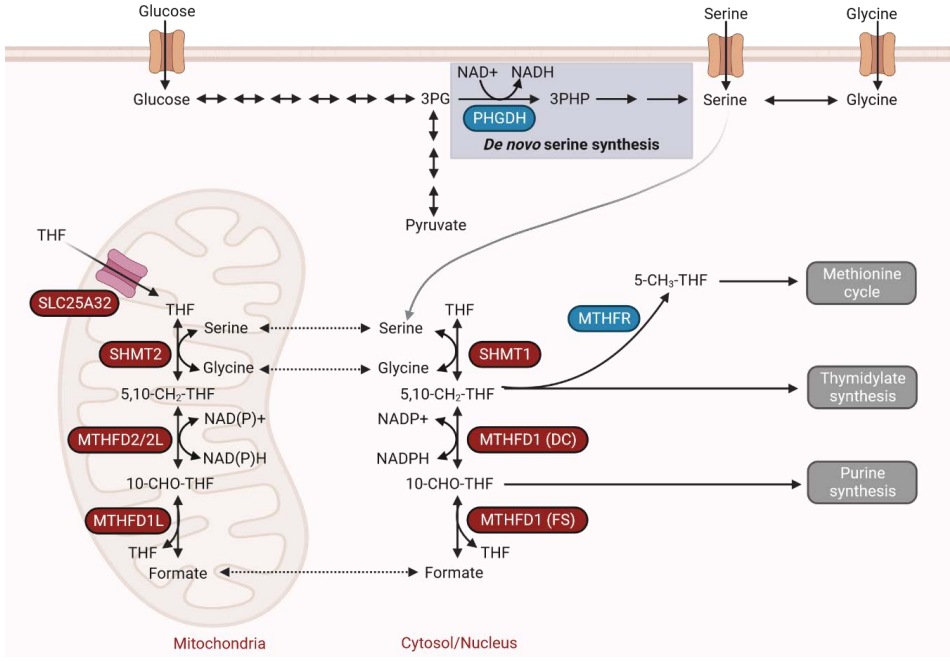

Figure 4. Schematic of the 1C folate pathway. Enzymes of the 1C folate cycle are highlighted in red. THF acts as a carrier of 1C units, and cytosolic THF enters mitochondria through the folate transporter SLC25A32. The majority of the 1C units are sourced from serine, which can be synthesized *de novo* from the glycolytic or gluconeogenic intermediate 3-phosphoglycerate (3PG) or taken up from the extracellular matrix. The *de novo* serine synthesis pathway consists of three enzymatic steps and the first, rate-limiting step is catalyzed by 3-phosphoglycerate dehydrogenase (PHGDH), which yields 3-phosphohydroxypuruvate (3PHP). Serine can enter either the mitochondrial or the cytosolic branch of the 1C folate cycle, which ultimately yields intermediates for thymidylate and purine synthesis, as well as methylation reactions. Dashed lines illustrate transport over the mitochondrial membrane. Image created with BioRender.com.

In addition to their mitochondrial and cytosolic localizations, one-carbon folate enzymes are also found in the cell nucleus, where they can support local *de novo* dTMP synthesis[83,85]. During the S-phase of the cell cycle or upon DNA damage, SHTM1, SHMT2α (an alternatively spliced isoform of SHMT2), MTHFD1, thymidylate synthase (TYMS) and DHFR are modified post-translationally by the small ubiquitin-like modifier (SUMO)[86–90]. This SUMOylation enables their translocation into the nucleus and formation of a multienzyme complex. SHTM1 and SHMT2α tether the complex to the nuclear lamina at replication sites[88]. Either SHMT enzymes or MTHFD1 can produce 5,10-CH_2-THF from serine or formate, respectively, which is required for dTMP synthesis[88,89]. The majority of 1C units donated to dTMP synthesis are derived from formate by the activity of MTHFD1[91–93]. Impaired nuclear MTHFD1 activity leads to reduced rates of *de novo* dTMP synthesis and excessive misincorporation of uracil into DNA, followed by DNA single-strand breaks during base excision repair, which may be converted to DNA double-

strand breaks during replication[94–96]. In addition to promoting nuclear thymidylate synthesis and genome integrity, MTHFD1 has recently been shown to regulate transcription in cancer by interacting with the histone acetyl reader BRD4 and to bind to chromatin[97].

The mitochondrial enzyme MTHFD2 has also been shown to reside in the nucleus and co-localize with replication sites[98]. Since MTHFD2 does not have formyltetrahydrofolate synthetase activity, it cannot convert formate to 5,10-CH_2-THF. Moreover, MTHFD2 is a NAD-dependent enzyme thought to mainly oxidize 5,10-CH_2-THF to 10-CHO-THF, making it unlikely that the enzymatic activity of MTHFD2 would contribute to nuclear dTMP synthesis[99]. Consequently, this would suggest a non-enzymatic function of MTHFD2 in the nucleus. Interestingly, MTHFD2 was recently reported to support non-homologous end joining (NHEJ) repair in the nucleus of p53-deficient colorectal cancer cells and interact with the NHEJ repair factor PARP3, independent of its catalytic activity[100]. The exact molecular mechanisms behind the nuclear role of MTHFD2 in relation to DNA replication and repair provide interesting avenues for future, more detailed characterization.

1.2.3 Utilization of mitochondrial versus cytosolic folate pathways

In nutrient-rich conditions, many but not all cancer cells utilize the mitochondrial folate pathway to generate 1C units. Interestingly, loss of the mitochondrial pathway can be compensated by a reversal in the cytosolic flux, highlighting the plasticity of folate metabolism branches and functional redundancy[101]. Double deletion of SHMT1/SHMT2 or SHMT1/MTHFD2 were previously shown to effectively block growth of xenografts in mice, whereas single deletions only modestly inhibited cell proliferation under nutrient-rich conditions[101–103]. This suggests that despite many 1C folate enzymes being highly overexpressed in cancers, inhibition of multiple enzymes might be essential to effectively halt cancer growth[62].

A recent report by Lee and co-workers published in 2021 showed that intracellular folate availability dictates tumor dependence on cytosolic versus mitochondrial 1C metabolism, which in turn is regulated by SLC19A1 expression[104]. In medium with physiological folate concentrations, cells were shown to rely on the cytosolic 1C pathway through SHMT1, suggesting SHMT1 as an attractive anticancer drug target in low SLC19A1-expressing tumors[104]. However, this study also found great differences between different cancer cell lines cultured at physiological folate levels in regard to the utilization of the mitochondrial or cytosolic folate pathway. These findings thus emphasize the fact that the direction of the 1C flow at a given folate concentration is likely cell-type dependent.

1.2.4 MTHFD1 and MTHFD2 in cancer

Of all the 1C folate metabolism enzymes, MTHFD2 has recently gained a lot of attention in cancer research. A meta-study of available gene expression data highlighted MTHFD2 as the most frequently overexpressed metabolic gene across different human tumors, and the MTHFD2 protein was also shown to be particularly expressed in cancer cells but not in the neighboring stromal cells[47]. In mitochondria, the conversion of 5,10-CH_2-THF to 10-CHO-THF can be catalyzed by two isoenzymes, MTHFD2 and MTHFD2L. Interestingly, MTHFD2 activity is important during early embryogenesis, as well as in undifferentiated and transformed cells[102,105–107], whereas MTHFD2L is mostly absent at early embryonic stages, but widely expressed in healthy adult tissue[108,109]. Although both isozymes are expressed in highly proliferative tissues and cancer cells, MTHFD2 generally appears to drive rapid proliferation and shows higher expression, as well as a more striking response to stimulation with growth factors compared to MTHFD2L[98,110]. Thus, MTHFD2L does not appear to play a pivotal role in supporting increased proliferation or tumorigenesis[110].

The frequent reactivation of MTHFD2 in cancers suggests a likely isoform switch from MTHFD2L to MTHFD2, encouraging development of MTHFD2 inhibitors for cancer treatment. Overexpression of MTHFD2 is linked to an overall poor prognosis in colorectal and breast cancer, as well as in renal cell and hepatocellular carcinoma patients[111–114]. MTHFD2 expression has also been shown to be linked to breast cancer cell migration and invasion[115]. In addition, loss of MTHFD2 protein was sufficient to impair cancer growth in mouse models of acute myeloid leukemia (AML)[116]. Indeed, MTHFD2 has been widely validated as a promising anticancer target in various solid and hematological malignancies during the past decade[117].

The cytosolic MTHFD1 enzyme is also associated with cancer, although it has generally been less extensively investigated as an emerging anticancer target compared to MTHFD2. The MTHFD1 protein is expressed in both cancer and in healthy tissue[118], especially in the liver[119]. Studies of available gene expression data have previously shown that MTHFD1 is upregulated in several different human cancers[45,47], suggesting that it may play a role in tumorigenesis. Overexpression of MTHFD1 correlates with overall poor survival and cancer recurrence in hepatocellular carcinoma[120], lung cancer[121] and cholangiocarcinoma patients[122]. MTHFD1 has also been identified to suppress anoikis and promote tumor distant metastasis in esophageal squamous cell carcinoma cells[123]. Moreover, depletion of MTHFD1 decreased the number of circulating melanoma cells in the blood and reduced metastatic disease burden in melanoma patient-derived xenograft mouse models[124]. MTHFD1 has also been proposed to contribute to lower intracellular levels of reactive oxygen species (ROS) and gemcitabine resistance in cholangiocarcinoma[122]. Interestingly, MTHFD1 was also shown be recruited to chromatin by the histone acetyl reader BRD4 and to control transcription in cancer cells, suggesting a link between 1C folate metabolism and regulation of gene expression[97].

In summary both MTHFD1 and MTHFD2 are linked to cancer cell proliferation, migration and metastasis in various cancer models.

1.2.5 Recent advances in therapeutic targeting of one-carbon folate metabolism in cancer

Since Sidney Farber's initial discovery of antifolates in 1948[21], followed by the development of methotrexate[65], as well as identification of TYMS as the main molecular target of fluorinated pyrimidines[125], pharmacological targeting of folate-mediated 1C metabolism has been recognized as a therapeutic strategy to treat cancer. However, methotrexate, for instance, also targets many other folate enzymes in addition to its primary target DHFR, which causes undesired toxicities in non-transformed tissues, such as intestinal epithelium and bone marrow[62]. Currently, there is a great interest to develop more selective inhibitors of the 1C folate metabolism which hold the potential to halt tumor progression with less adverse effects.

Recently, selective SHMT inhibitors (SHIN1, SHIN2, AGF347) targeting both the cytosolic SHMT1 and mitochondrial SHMT2 have been developed[103,126–128]. SHIN2 exhibits *in vivo* anticancer efficacy in mouse primary T-cell acute lymphoblastic leukemia (T-ALL), as well as in patient-derived T-ALL xenograft model[126]. Work on SHMT inhibitors also indicates that simultaneous inhibition of the cytosolic and mitochondrial folate cycles may be necessary to counteract metabolic plasticity within the 1C folate pathway and effectively block the production of 1C units from serine.

Due to its cancer-specific expression profile[47], MTHFD2 is an attractive target for inhibitor development. However, development of selective MTHFD2 inhibitors has been challenging and spanned over 40 years[129]. MTHFD2 shares 40 % sequence identity with MTHFD1[82], and the fact that there are only a few residues in the substrate binding site that vary between MTHFD1 and MTHFD2 poses a challenge for inhibitor development[130]. Other challenges for MTHFD2 inhibitor development is drug delivery into the mitochondria and different chemical conditions inside the mitochondria as compared to the cytosol[131]. Yet another challenge comes with potential compensatory metabolic changes in cytosolic 1C flux[101,131,132]. Given the crosstalk between the cytosolic and mitochondrial folate pathways, a dual inhibition of MTHFD1 and MTHFD2 might be necessary for effective cancer therapy[62,132]. This remains to be evaluated in the future, and the advantages of dual inhibition need to be weighed against potential toxicities. LY354899 was among the first MTHFD inhibitors to be developed and has been shown to inhibit mainly MTHFD1, but also MTHFD2 to a lesser extent[130,133–135]. This inhibitor has also been reported to exhibit anti-tumor activity *in vivo* in colorectal xenograft models, but the extent of the individual contributions of MTHFD1 and MTHFD2 inhibition remain unclear[112]. Yet another MTHFD2 inhibitor is the recently disclosed DS18561882, which has

been shown to impair tumor growth in an MDA-MB231 mouse xenograft model after oral administration[136].

In conclusion, 1C folate metabolism provides new, interesting anticancer targets, but the path for clinical development of novel 1C metabolism inhibitors may be challenging.

1.2.6 *De novo* thymidylate biosynthesis

The *de novo* thymidylate synthesis occurs in the mitochondria[137] and in the nucleus[86,92,138–140] to support DNA replication. Deoxythymidine monophosphate (dTMP or thymidylate), which is the precursor of deoxythymidine triphosphate (dTTP), is synthesized *de novo* from deoxyuridine monophosphate (dUMP)[141] (Figure 1). In summary, in the *de novo* thymidylate synthesis pathway, the pyrimidine ring is first synthesized as uracil. Initially, six enzymatic steps (Figure 5) in the cytosol and mitochondria convert glutamine, bicarbonate, aspartate and PRPP to uridine monophosphate (UMP)[142]. The resulting UMP is then phosphorylated by cytidine monophosphate kinase (CMPK) to yield uridine diphosphate (UDP). The RNR enzyme reduces UDP into deoxyuridine diphosphate (dUDP), which in turn is converted to deoxyuridine triphosphate (dUTP) by the NDPK enzyme (Figure 1).

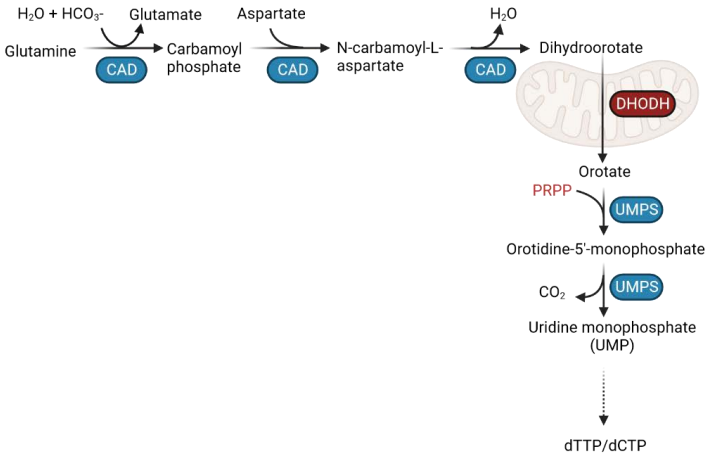

Figure 5. The first six enzymatic steps in the *de novo* pyrimidine synthesis pathway. Cytosolic enzymes are colored in blue, while the mitochondrial membrane protein dihydroorotate dehydrogenase (DHODH) is highlighted in red. Dashed arrow represents multiple enzymatic steps leading to generation of dTTP and dCTP. CAD, carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase; UMPS, uridine monophosphate synthetase; HCO_3-, bicarbonate; PRPP, phosphoribosyl pyrophosphate. Image created with BioRender.com.

Following either the hydrolysis of dUTP into dUMP and pyrophosphate by the enzyme dUTP diphosphatase (dUTPase), or the deamination of deoxycytidine monophosphate (dCMP) to yield dUMP by deoxycytidylate deaminase (DCTD), dUMP and the 1C folate intermediate 5,10-CH_2-THF are converted to dUMP in one enzymatic step catalyzed by

TYMS[54] (Figure 6). This reaction also generates DHF that needs to be reduced back to THF by DHFR to allow subsequent cycles of the thymidylate biosynthesis and avoid folate trapping as DHF. Ultimately, deoxythymidylate kinase (DTYMK) and NDPK phosphorylate dTMP to dTDP and ultimately to dTTP (Figure 1).

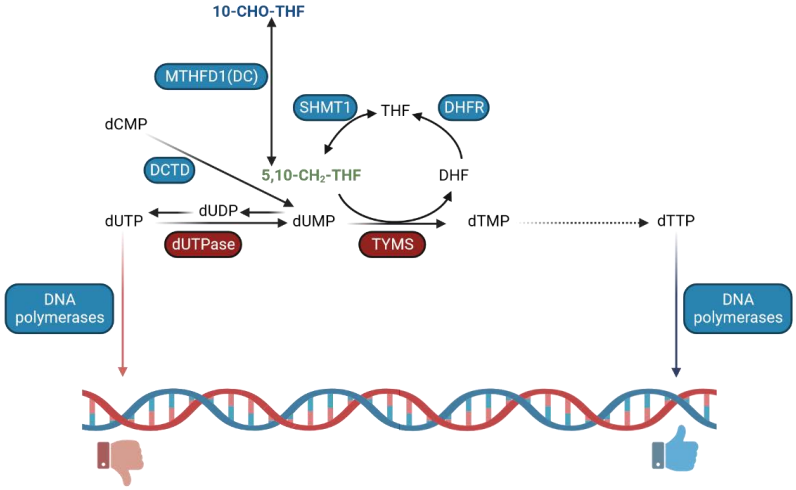

Figure 6. One-carbon metabolism fuels thymidylate synthesis. 5,10-methylene-THF (5,10-CH_2-THF, highlighted in green) and dUMP are converted into thymidylate (dTMP) and DHF by TYMS. To regenerate free THF, DHF is reduced by DHFR, making it available for the 1C folate cycle again. Thymidylate is required for synthesis of dTTP which is used as a DNA polymerase substrate and is correctly incorporated into DNA. If not used by TYMS, dUMP can also give rise to dUTP. DNA polymerases readily catalyze incorporation of dUTP into DNA, leading to incorrect DNA synthesis and genome instability. To promote high fidelity DNA synthesis, the enzyme dUTPase limits excessive accumulation of dUTP by hydrolyzing dUTP into dUMP. Dashed line represents several enzymatic steps. Image created with BioRender.com.

Importantly, deregulation of cellular dTTP pools leads to increased misincorporation of uracil into DNA, followed by mitochondrial dysfunction[143] and nuclear genome instability[94,144]. Some chemotherapeutic agents targeting *de novo* thymidylate synthesis, such as 5-fluorouracil (5-FU), have earlier been shown to induce an imbalance of cellular dUTP/dTTP ratio as a part of their mechanism of action[145]. Most eukaryotic DNA polymerases are not capable of discriminating between dUTP and dTTP[146], thus incorporating either one of them based on their relative abundance[147] (Figure 6). Both thymidine and uracil can readily base-pair with adenine. To prevent excessive misincorporation of uracil during DNA replication, the dUTP/dTTP ratio is under tight regulation and kept low[148,149]. For instance, the hydrolysis of dUTP to dUMP by dUTPase represents one of the major mechanisms for cells to decrease their intracellular dUTP pools (Figure 6) and thus prevents incorporation of dUTP into DNA during replication and repair[150,151]. Importantly, poor clinical response to the TYMS inhibitors 5-FU and 5-fluorodeoxyuridine (FUdR) in cancer patients correlates with elevated dUTPase levels,

and upregulation of dUTPase expression contributes to the development of drug resistance[152–154]. Thus, dUTP metabolism seems to play a crucial role in the anti-cancer activity of drugs targeting thymidylate biosynthesis through inhibition of TYMS. Moreover, the nuclear localization of not only dUTPase, but also TYMS, DHFR, and the 1C metabolism enzymes SHMT1, SHMT2α, MTHFD1 and MTHFD2 illustrate the significance of local *de novo* thymidylate synthesis near the sites of active DNA replication and repair to limit dUTP accumulation[88,89,98,138,151,155].

1.2.7 *De novo* purine synthesis

In addition to pyrimidine nucleotides, cells also need purines for DNA and RNA synthesis. Moreover, purine nucleotides play a crucial role in intracellular signaling and provide energy for metabolism. Under conditions requiring higher purine levels, such as in cancer cells, purine salvage is often insufficient to meet this increased cellular demand[57,156]. Therefore, the *de novo* purine synthesis is required to restore the cellular purine pool under high purine nucleotide needs[157]. *De novo* purine synthesis takes place in the cytosol and converts PRPP into inosine 5′-monophosphate (IMP), which acts as a precursor for AMP and GMP. In most eukaryotes, the *de novo* purine pathway comprises ten enzymatic steps catalyzed by six different enzymes (Figure 7)[158,159].

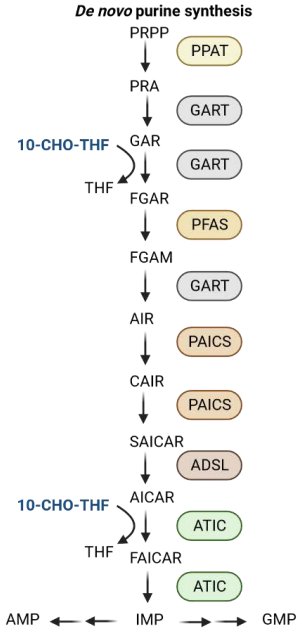

Figure 7. Schematic of the eukaryotic *de novo* purine synthesis pathway. Co-factors derived from the 1C folate metabolism are highlighted in blue. Image created with BioRender.com.

The *de novo* purine synthesis starts with the presumably rate-limiting reaction where PRPP is converted to 5-phosphoribosylamine (PRA) by PRPP amidotransferase (PPAT; Figure 7)[57]. Thereafter, the trifunctional enzyme phosphoribosylglycinamide

formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase (GART) adds glycine and a formyl group derived from 10-CHO-THF onto PRA to produce *N*-formylglycinamide ribonucleotide (FGAR). Phosphoribosyl formylglycinamidine synthase (FGAMS) then catalyzes conversion of FGAR into *N*-formylglycinamidine (FGAM), which is subsequently transformed into aminoimidazole ribonucleotide (AIR) by the phosphoribosylaminoimidazole synthetase activity of GART. The bifunctional phosphoribosylaminoimidazole carboxylase and phosphoribosylaminoimidazole succinocarboxamide synthase (PAICS) converts AIR to N-succinocarboxyamide-5-aminoimidazole ribonucleotide (SAICAR), followed by formation of 5-amonoimidazole-4-carboxamide ribonucleotide (AICAR) catalyzed by adenylosuccinate lyase (ADSL). The remaining two steps require a formyl group derived from 10-CHO-THF and yield IMP. This is catalyzed by the bifunctional enzyme 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC)[57].

Since many of the *de novo* purine pathway intermediates are fairly instable, it has long been hypothesized that a physical complex formation between the different *de novo* purine enzymes, a metabolon, would allow enzyme proximity, substrate channelling and thus more efficient metabolic flux[160,161]. Previously, intracellular fluorescent imaging revealed that these proteins indeed cluster around mitochondria and microtubules under purine-depleted conditions to form a transient, multienzyme complex, termed the purinosome[162–170]. Three enzymes, PPAT, GART and FGAMS form the core scaffold, whereas PAICS, ADSL and ATIC constitute peripheral proteins[57]. Adenylosuccinate synthase (ADSS) and inosine monophosphate dehydrogenase (IMPDH) were also identified to participate in the purinosome formation[166]. Mutations in ADSL and ATIC genes were shown to result in impaired purinosome formation in skin fibroblasts, indicating that even the activity of the peripheral purinosome enzymes influences complex stability[164]. Moreover, depletion of GART, ADSL, ATIC, PAICS and FGAMS was reported to significantly reduce the number of purinosomes[167].

Interestingly, purinosomes were found to co-localize with mitochondria in an mTOR-dependent manner[168]. Mitochondrial 1C metabolism enzymes convert serine into glycine and formate which act as substrates for the *de novo* purine synthesis. Recently, metabolic profiling provided evidence for the notion that the end products of purine synthesis, AMP and GMP, are synthesized mainly from mitochondrially generated substrates, thus indicating direct channeling of mitochondrial formate to purinosomes[171]. MTHFD1 supplies the essential 10-CHO-THF cofactor for the *de novo* purine synthesis and it has therefore been suggested to participate in the purinosome[171]. To strengthen this hypothesis, PAICS was shown to mediate purinosome formation and interact with MTHFD1 in intact HeLa cells[172]. Taken together, these findings suggest a close crosstalk between 1C folate metabolism and *de novo* purine synthesis.

1.3 Genomic instability, altered metabolism and cancer

Every day, throughout the entire lifespan of a cell, DNA damage caused by both endogenous and exogenous sources threatens the genomic stability of the cell. Endogenous sources of DNA damage arise from physiological processes, such as DNA replication, oxidative respiration and redox reactions which generate reactive oxygen species (ROS). Exogenous insults include DNA-damaging agents such as ionizing radiation (IR), ultraviolet (UV) light, carcinogenic chemicals like benzo[a]pyrene, and chemotherapeutics used to treat cancer[4,173,174]. To preserve genomic integrity, cells have developed diverse systems to sense DNA damage, indicate its presence and promote subsequent repair or cell death. This genome maintenance network is collectively termed as the DNA damage response (DDR) and it includes proteins that sense the DNA lesion, the PI3 kinase-related kinases (PIKKs) that participate in signaling of the damage (ATR, ATM and DNA-PK; Figure 8) and downstream DNA repair proteins[4,175]. Mutations drive tumor initiation and progression, and genomic instability is considered as a hallmark of cancer[9,176]. Cancer cells often exhibit defects in one or several DDR pathways, and they are characterized by increased levels of genomic instability. Although perturbed DDR enables tumor development and plasticity, the subsequent increased genetic instability also acts as a double-edged sword, rendering cancer cells more reliant on a limited repertoire of genome maintenance pathways for survival. Therefore, defective DDR and DNA repair are potential targets for pharmacological intervention[177–180].

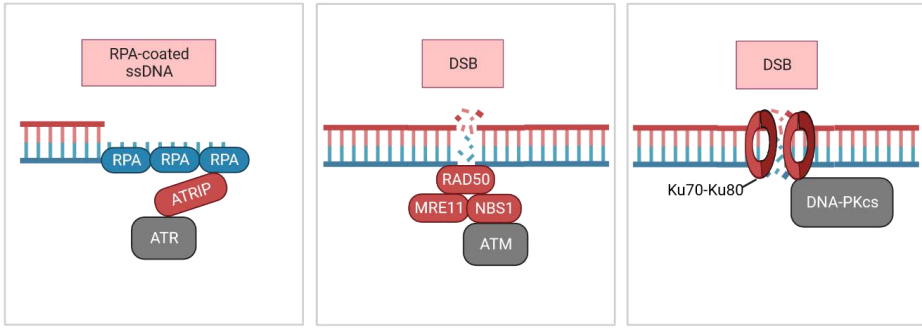

Figure 8. Recruitment of PI3K-related kinases ATR, ATM and DNA-PKcs which act as the main signal transducers in DDR upon DNA damage. The specific protein co-factors ATRIP, the MRN complex (MRE11-RAD50-NSB1) and the Ku70-K80 heterodimer regulate the recruitment and activation of ATR, ATM and DNA-PKcs, respectively, after DNA damage and are highlighted in red. ssDNA, single-stranded DNA; RPA, replication protein A; DSB, double strand break. Image created with BioRender.com.

1.3.1 Replication stress response

To keep the genetic information intact from one cell division to another and to avoid malignant transformation, cells need to faithfully duplicate their entire genome during

DNA replication. Although replication is highly regulated to ensure high fidelity, replication forks can encounter various obstacles that hinder their movement along the DNA template. Slowing down, stalling or collapse of DNA replication forks due to these obstacles is defined as replication stress (RS). Importantly, RS is the primary cause of genome instability in cancer[181]. RS can be a transient or a persistent phenomenon. Prolonged RS can lead to collapsed replication forks, giving rise to DNA double-strand breaks (DSBs), the repair of which depends predominantly on the error-free homologous recombination (HR) pathway (Chapter 1.3.2)[181]. Many sources can lead to RS, including oncogene activation, different DNA lesions, difficult to replicate sequences, physical barriers that directly restrict replication fork progression (e.g., collisions between replication and transcription machineries and R-loop formation) and shortage of factors required for DNA synthesis[182,183]. For example, perturbations in the cellular dNTP pools cause RS and extended dNTP deprivation causes fork collapse[184–188]. Many anticancer treatments, including chemotherapeutic agents and DDR inhibitors, cause excessive RS by inducing DNA damage and interfering with replication[189].

Cells have evolved elaborate mechanisms to protect replication forks against RS and thus promoting faithful genome duplication. Upon stalling of replication forks, long stretches of single-stranded DNA (ssDNA) are formed due to uncoupling of MCM helicase from DNA polymerases[190]. This activates the RS response, or S phase checkpoint, which is orchestrated by the ataxia telangiectasia and Rad3-related kinase (ATR). First, to protect ssDNA from nucleolytic degradation and secondary structure formation, the replication protein A (RPA) complex rapidly coats it. This complex consists of three subunits, RPA70, RPA32 and RPA14. Thereafter, ATR is recruited by its partner protein ATR-interacting protein (ATRIP) to the RPA-coated ssDNA[191–193] (Figure 8). It is noteworthy that ATR signaling is not activated only by helicase-polymerase uncoupling at stalled replication forks, but also by nucleolytic processing of damaged DNA that generates RPA-coated ssDNA[194]. In addition to its recruitment to RPA-ssDNA, ATR kinase activity and autophosphorylation is further enhanced by physical interaction with ATR activators, such as DNA topoisomerase 2-binding protein 1 (TOPBP1) or Ewing Tumor-associated Antigen 1 (ETAA1)[195–199]. TOPBP1 recruitment to stalled forks relies on its interaction with RAD9, which is part of the ring-shaped 9-1-1 clamp complex loaded onto RPA-ssDNA/dsDNA junctions and consists of RAD1 and HUS1 proteins, in addition to RAD9[200–202]. ETAA1, in turn, binds directly to RPA coating ssDNA.

Once fully activated, ATR phosphorylates several downstream targets, such as RPA, histone variant H2AX (yielding γH2AX) and the effector checkpoint kinase 1 (CHK1)[183,203,204]. Phosphorylation of RPA further increases its affinity for ssDNA, acts as a signal for switching from replicative DNA synthesis to reparative DNA synthesis and recruits other DDR factors (e.g., BRCA2) to stalled forks[204–206]. Overall, activation of the ATR-CHK1 checkpoint leads to cell-protective events, including reduced global origin

firing to ensure sufficient RPA pools for local protection from fork breakage, increased dNTP production via the upregulation of the RRM2 subunit of RNR, recruitment of DNA repair factors and cell cycle arrest by reduced cyclin-dependent kinase (CDK) activity[181,183,207–209]. Thus, the ATR-CHK1 signaling pathway plays a pivotal function in ensuring successful completion of DNA replication and preventing replication fork breakage[181]. Recently, inhibitors of the ATR-CHK1 checkpoint have been developed to target cancer cells with high levels of RS[210].

1.3.2 Repair of DNA double-strand breaks (DSBs)

Among the different classes of DNA lesions, DSBs, although less frequent, are the most toxic type of DNA damage. DSBs can cause chromosomal rearrangements, breakage and cell death, if they are left unrepaired[211,212]. For instance, ionizing radiation (IR) used in radiation therapy induces DSBs, as well as other lesions such as DNA single-strand breaks (SSBs)[4]. A major endogenous source of DSBs is fork stalling and collapse during DNA replication[213]. Repair of DSBs poses a challenge to the cell since in this case both DNA strands are damaged. This means that there is no template available containing sequence information for correct repair[213]. To maintain genomic integrity, organisms have developed several mechanisms to repair DSBs as part of the DDR and these can be subdivided into homology-dependent and homology independent mechanisms.

The two most prominent cellular pathways responsible for DSB repair are homologous recombination (HR) and non-homologous end joining (NHEJ) repair (Figure 9). HR requires a sister chromatid as a template for repair synthesis, and therefore occurs in the S and G2 phases of the cell cycle. NHEJ, on the contrary, does not utilize a sister chromatid, but directly ligates DSB ends and can operate throughout the cell cycle. This process, however, is potentially error-prone[4,213–215]. The initiation of 5′-3′ end resection commits cells to HR repair and restricts NHEJ[216]. Tumor suppressor p53-binding protein 1 (53BP1) and breast cancer type 1 susceptibility protein (BRCA1) have particularly crucial functions in regulating the choice of DSB repair pathway. For instance in the G1 phase, 53BP1 and its effector protein RIF1 antagonize BRCA1-dependent end resection of DSBs and recruitment of BRCA2, preserving DSB ends and therefore promoting NHEJ repair over HR[217,218].

In mammalian cells, DSBs are repaired predominantly by NHEJ, except for DSBs occurring at collapsed replication forks, which are preferentially repaired via HR[191,219–221]. Faced with a large number of DSBs, NHEJ can lead to the loss of genetic material or chromosomal rearrangements[221]. In the classical NHEJ (c-NHEJ) pathway, the regulatory Ku (Ku70-Ku80) heterodimer recognizes the DSB and recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), thereby activating it (Figure 9)[222–224]. Ku and DNA-PKcs form a synaptic complex which mediates the recruitment and activation of end-processing enzymes, such as the endonuclease Artemis[225] and X family

polymerases (Pol μ, λ)[226], as well as proteins like DNA ligase IV (LIG4), X-ray repair cross-complementing protein 4 (XRCC4) and XRCC4-like factor (XLF), which are required for ligation of the DNA ends[227]. In addition to c-NHEJ, a Ku-independent NHEJ pathway exists, termed alternative NHEJ (alt-NHEJ) which utilizes a different set of repair factors. This pathway requires microhomology ranging between 2 to 20 base pairs (bp), as well as the activity of poly(ADP-ribose) polymerase 1 (PARP1) and DNA polymerase θ (Pol θ)[221].

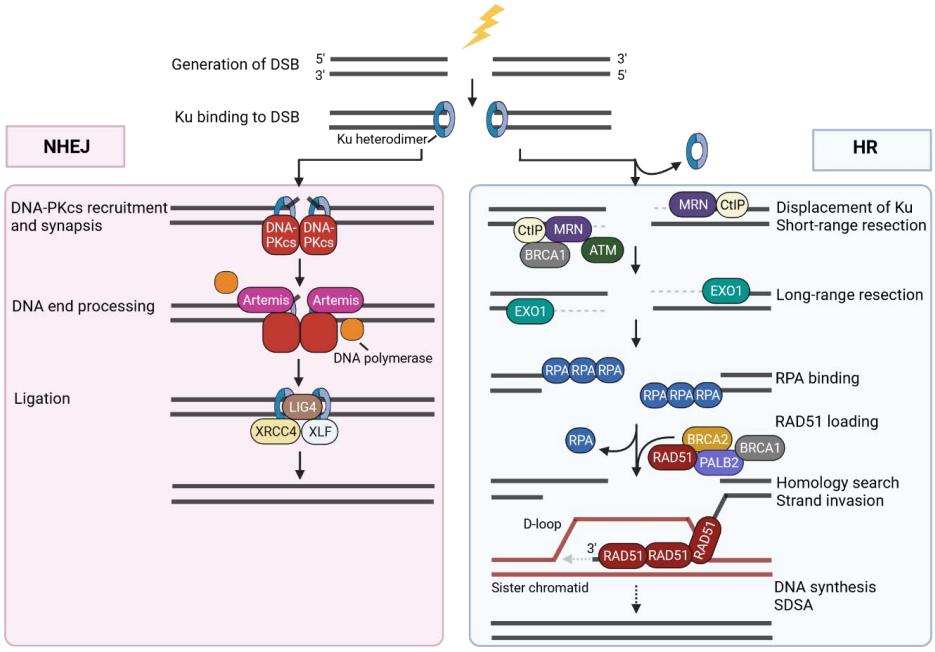

Figure 9. Simplified schematic of the two major DSB repair pathways. The Ku heterodimer is abundantly expressed throughout the cell cycle[238] and rapidly binds free DNA ends following DSB formation. Recruitment of DNA-PKcs by Ku initiates NHEJ repair pathway and synapsis, i.e., juxtaposition of the DNA ends. If required, DNA ends can be processed by Artemis and DNA polymerases (μ, λ). Lastly, the LIG4-XRCC4-XLF complex ligates the DNA ends to complete repair. In HR repair, the MRN complex binds to DNA and initiates short-range end resection at Ku-blocked DNA ends, resulting in removal of Ku[239]. It also recruits ATM which signals the damage and phosphorylates several target proteins, such as BRCA1. The activity of the MRN complex is further enhanced by CtIP and BRCA1. To initiate long-range end resection, the MRN complex loads EXO1 onto the DNA ends. RPA coats the ssDNA formed during resection, but is later exchanged to RAD51 by the BRCA2-PALB2-BRCA1 complex. Following homology search on the sister chromatid, RAD51 initiates repair synthesis by positioning the invading 3' end on a template DNA. Completion of HR involves DNA synthesis, as well as synthesis-dependent strand-annealing (SDSA) which are not detailed here. PALB2, partner and localizer of BRCA2. Image created with BioRender.com from information in references[223,240].

Besides NHEJ, DSBs can also be repaired by the HR pathway which is considered to be an error-free mechanism (Figure 9). HR is a stepwise process involving DNA end resection, strand invasion, DNA synthesis and resolution. Upon DSB formation, the MRE11-RAD50-NBS1 (MRN) complex serves as a damage sensor that rapidly recruits and activates the ataxia telangiectasia mutated (ATM) kinase[228–233]. ATM in turn phosphorylates the tail of the histone variant H2AX at serine 139, which yields γH2AX flanking the DSB[234]. γH2AX signals for the accumulation of downstream repair factors[235,236] and promotes the recruitment of the mediator of DNA damage checkpoint protein 1 (MDC1)[237]. MDC1 acts as a scaffolding protein further recruiting MRN to the damage site. MDC1-MRN then further enhance the chromatin-recruitment and activation of ATM, thereby amplifying the DNA damage signaling[191].

During HR repair, the endonuclease activity of MRE11 in the MRN complex, together with the CtBP-interacting protein (CtIP), initiates DNA end resection. This activity represses NHEJ by promoting the removal of Ku70-Ku80 from the DNA ends[233] (Figure 9). BRCA1 also facilitates initial end resection by interacting with MRN and CtIP[223,240]**.** Next, the MRN complex promotes recruitment and processivity of exonuclease 1 (EXO1), which catalyzes 'long-range' resection[223,233]. This creates long 3' stretches of single-stranded DNA (ssDNA) which is rapidly coated by RPA. Subsequently, RPA becomes replaced by the recombinase RAD51 in a BRCA1/2-dependent process[241]. Genetic or epigenetic inactivation of BRCA1/2 genes is a main source of HR deficiency and contributes to the development of many cancers[242]. RAD51 conducts homology search on the sister chromatid and catalyzes subsequent strand invasion with the assistance from other repair factors, such as the BRCA1-BARD1 complex that enhances the recombinase activity of RAD51[243–246]. The invading strand and template DNA form a D-loop structure[247], followed by DNA synthesis on the invading strand catalyzed by DNA polymerases δ and ε. Finally, the D-loop structure is resolved predominantly by synthesis-dependent strand-annealing (SDSA) in somatic cells, which does not lead to cross-over and thereby preserves heterozygosity[223,240].

There is an intricate interplay between DNA repair mechanisms and cellular metabolism. Although metabolic activity can indirectly generate DNA damage (e.g. ROS), it also supports DNA repair[248]. Especially in cancer cells, metabolic reprogramming increases DNA repair capacity, thus protecting tumor cells against DNA damaging radiation and chemotherapy[249]. Recently, many metabolic enzymes have been identified to support DSB repair by regulating dNTP pools, interacting directly with DNA repair factors or modulating epigenetic remodeling[248]. Recruitment of ribonucleotide reductase and thymidylate kinase to DNA damage sites highlights the need to synthesize dNTPs locally during DNA repair[208,250,251]. Previously, together with Dr. Gustafsson, we demonstrated that the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) translocates to DNA repair foci and recruits RRM2, a subunit of RNR to support

HR repair in cancer cells[252]. Another glycolytic enzyme, pyruvate kinas M2 (PKM2) was also shown to display moonlighting functions in the nucleus and promote HR-mediated repair of DSBs by phosphorylating γH2AX and CtIP, thereby contributing to the activation of DSB signaling and end resection[253,254]. The 1C folate metabolism enzyme MTHFD2 also localizes to the nucleus[98] and has been suggested to support HR through altering EXO1 activity by cyclin-dependent kinase-1 (CDK1) in mouse pluripotent stem cells[255], as well as NHEJ repair by a physical interaction with PARP3 in p53-deficient colorectal cancer cells[100]. Moreover, the *de novo* purine synthesis enzyme PAICS was recently suggested to support both HR and NHEJ repair, interact with histone deacetylases 1/2 (HDAC1/2) and promote RAD51 recruitment to cisplatin-induced DNA-damage sites in gastric cancer cells[256].

In conclusion, metabolic reprogramming enhances DNA repair and cancer cell survival, and pharmacological targeting of metabolic enzymes emerges as a potential strategy to combat cancer and sensitize cancer cells to radiation or chemotherapy.

2 Research aims

The overall aim of this thesis was to examine new inhibitors targeting the 1C folate metabolism enzymes MTHFD1 and MTHFD2 in cancer, as well as study the emerging nuclear role of MTHFD2 in the DNA damage response. These objectives were addressed in the constituent papers and the specific goals per paper were:

Paper I: Pharmacological targeting of MTHFD2 suppresses acute myeloid leukemia by inducing thymidine depletion and replication stress

- Validate MTHFD2 as an anticancer target
- Determine how MTHFD2 affects replication stress
- Develop potent, cell-active small-molecule MTHFD2 inhibitors
- Characterize the mechanism of action of the newly developed inhibitor series
- Determine the antitumor efficacy of MTHFD2 inhibitors *in vivo*
- Examine potential synergy between MTHFD2 and DDR inhibitors in cancer cells

Paper II: Formate overflow drives toxic folate trapping in MTHFD1 inhibited cancer cells

- Revisit the mechanism of action of the MTHFD1/2 inhibitor (MTHFD1/2i) TH9619 in cancer
- Determine direct inhibition of mitochondrial MTHFD2 by different MTHFD1/2i
- Assess the importance of targeting MTHFD1(DC) for TH9619 toxicity
- Compare the biological effects of MTHFD1/2i in *MTHFD2* wildtype and knockout cells
- Determine accumulation of 1C pathway intermediate 10-CHO-THF following MTHFD1/2i treatment in *MTHFD2* wildtype and knockout cells
- Compare the mechanism of action of TH9619 and related compounds with that of other 1C metabolism inhibitors and traditional antimetabolites

Paper III: Targeting MTHFD2 impairs homologous recombination and sensitizes cancer cells to PARP inhibitors

- Study the association of MTHFD2 with active replication forks
- Investigate the subcellular localization and nuclear recruitment of MTHFD2 upon induction of DSBs by etoposide, hydroxyurea or ionizing radiation (IR)
- Dissect the DDR signaling cascade following IR treatment to elucidate where MTHFD2 comes into play to promote DSB repair
- Assess the effect of MTHFD2 silencing on HR and NHEJ activity, as well as cell survival after IR treatment
- Investigate whether targeting MTHFD2 can generate a HR defect and sensitize HR-proficient cancer cells to PARP inhibitors

3 Materials and methods

This chapter presents some of the principal methods used in **Papers I-III**. A complete list of all experimental techniques, including detailed descriptions, can be found in the materials and methods section of each research paper.

3.1 Cellular target engagement assays

Evaluating small-molecule target engagement in intact cells allows validation of mechanism of action and intracellular efficacy of a compound and it is therefore a crucial part of the drug discovery process and chemical biology research[257]. To understand how MTHFD1/2 inhibitors engage their targets in cells, two label-free methods were used in **Paper I** and **Paper II**, and the basic principles of these assays are outlined below.

3.1.1 Cellular thermal shift assay (CETSA)

Proteins denature at increasing temperatures and each protein has its distinct melting temperature. The thermal stability of a protein can be influenced by binding of a small molecule, and this principle is exploited in thermal shift assays[258]. Ligand binding can result in a shift of protein melting temperature either due to thermal stabilization or destabilization of the protein target[257,258]. Cellular thermal shift assay (CETSA) follows the basic principle of traditional thermal shift assays, but it uses intact cells, cell lysates or even tissue samples (e.g., tumors) instead of purified proteins, thus allowing evaluation of target engagement in a more complex environment[259,260].

Today, there are several different formats of CETSA available, but they all typically include heating cells, which are treated with either a vehicle or a drug, at increasing temperatures to denature proteins. This is followed by precipitation of aggregated, denatured proteins by centrifugation and quantification of the remaining soluble proteins by Western blotting or mass spectrometry (MS)[259,260]. A classical CETSA format is the comparison between melting curves of a target protein in the presence and absence of a saturating concentration of a small molecule and it determines whether protein stabilization or destabilization occurs upon compound binding. This allows addressing the basic question of whether a compound engages its intended target inside the cell[259,260]. To determine the potency of a compound in cells, isothermal dose-response fingerprint (ITDRF) CETSA can be performed. In this format, cells are treated with varying concentrations of the compound and heated at a single temperature which denatures most of the target protein in the vehicle-treated sample[260].

One of the advantages of the CETSA methodology is that it allows studies of small molecule target engagement in a more biologically relevant context. Use of whole cells instead of purified proteins helps to preserve post-translational modifications, protein-

protein interactions and correct subcellular localization of proteins[260]. Moreover, drug target engagement can be effectively studied even in tumor tissues using CETSA, and this provides important information of whether the drug reaches the tumor[259]. Because CETSA methodology uses cells or tissues, it is, unlike other biophysical methods (e.g., surface plasmon resonance, differential scanning fluorimetry) not dependent on purification of recombinant proteins. CETSA also enables both confirmation and deconvolution of target biology and it is a relatively cheap technique which utilizes equipment that is often found in every biochemistry and molecular biology laboratory[257,259,260]. Data interpretation is relatively easy when immunoblotting is used for protein detection, whereas CETSA experiments coupled with MS require special equipment and skilled technical staff to interpret complex data[257]. Additionally, CETSA can be adapted for high-throughput formats[260].

Like all methods, CETSA also has several limitations. CETSA formats that use Western blotting as their primary detection method rely heavily on the availability of specific antibodies[257]. Moreover, not all proteins denature within the proposed temperature range, making CETSA less suitable for studying small, heat-resistant proteins[257]. Some proteins can also aggregate very rapidly after a certain temperature, thus yielding melting profiles that do not follow a sigmoidal shape. As an example, CETSA analysis of MTHFD1 stabilization was not successful in **Paper I** due to lack of good antibodies at the time of the study, as well as the rapid aggregation of MTHFD1 at increasing temperatures. Lastly, CETSA relies on the principle that the binding of a small molecule to its protein target induces significant thermal stabilization or destabilization of the investigated target. This is problematic especially in the case of large proteins where compound binding may stabilize/destabilize only a small part of the protein. Therefore, the binding of a small molecule does not necessarily alter the overall thermal stability of the protein and can thus result in false negatives[257,260].

3.1.2 Drug affinity responsive target stability (DARTS)

Drug affinity responsive target stability (DARTS) assay is an orthogonal method to CETSA. Like CETSA, the DARTS methodology is based on the principle that the binding of a ligand can induce conformational changes or stabilize the target protein. The method was originally developed to facilitate target identification, but it can also be used as a tool for target validation[261]. Instead of thermal denaturation, DARTS utilizes proteolytic degradation of proteins to assess target stabilization upon ligand binding. Target protein is stabilized upon small molecule binding which in DARTS is detected as binding-induced increase in resistance to proteolysis[261]. Pronase, which is a mixture of different proteases, is often used in DARTS experiments to degrade both folded and unfolded proteins due to its broader substrate specificity as compared to thermolysin, for instance[262].

DARTS can be performed on cell lysates, whole cells or even tissue samples, thus enabling target engagement studies in a biologically relevant model[261]. In a typical DARTS experiment, cells are treated with either a vehicle or a test compound, lysed and subjected to digestion with increasing concentrations of protease. To detect protein stabilization or destabilization upon ligand binding, a proteomic technique like Western blotting or liquid chromatography coupled to tandem mass spectrometry (LC/MS-MS) needs to be included in the experimental protocol after the proteolysis step[261–263].

DARTS shares many advantages with CETSA. For example, both methods are label-free and thus do not require modification of the ligand molecule or the target protein[261–263]. Moreover, they preserve biological relevance better than other biophysical methods that do not include complex protein mixtures. Both methods are also relatively inexpensive and straightforward. Interestingly, in this thesis, DARTS was deemed as the preferred method over CETSA when studying larger target proteins (**Paper II**), such as MTHFD1, which can rapidly aggregate at increasing temperatures.

However, DARTS experiments require careful optimization of the protease concentrations used during the proteolysis step when adapting the protocol for new protein targets in order to establish their susceptibility to proteolysis. In addition, some proteins, like stress proteins, may not be suited for DARTS analysis since they are naturally relatively resistant to protease digestion[261,264]. Moreover, this method also relies on the availability of highly specific antibodies if immunoblotting is used as the final detection method. Ligand binding can also indirectly influence the sensitivity of non-target proteins to proteolysis, especially if the primary drug target is part of a protein complex[261]. Therefore, this should also be considered when interpreting the results derived from DARTS experiments on whole cells. To better distinguish between direct and indirect effects of a ligand, performing additional data on cell lysates and biochemical assays should be carefully taken into account.

3.2 *In vitro* cell viability and survival assays

3.2.1 Resazurin cell viability assay

To estimate the number of viable cells following treatment with different inhibitors and/or metabolites, the resazurin assay was performed in **Papers I–III**. Resazurin is a cell-permeable, non-fluorescent blue dye that is enzymatically reduced to resorufin by accepting electrons from reducing agents such as NADH, NADPH and FADH in metabolically active, viable cells[265,266]. Resorufin has a pink color and it is highly fluorescent. Cells are normally incubated with resazurin for 1–4 hours to allow metabolically active cells to reduce resazurin into resorufin. The conversion of resazurin to resorufin can be measured in a standard microplate reader using 560 nm excitation/590 nm emission filter settings[265]. The amount of resorufin produced is proportional to the number of viable cells.

The resazurin assay is a simple, fast and inexpensive method and it can be easily adapted for high-throughput screens. Additionally, both adherent and suspension cells can be used in this assay[267]. Since it is a fluorometric assay, it offers increased sensitivity as compared to colorimetric tetrazolium reduction cell viability assays[265]. However, a drawback is the possible interference from test compounds containing certain functional groups with the resazurin assay even in the absence of metabolically active cells[268]. Therefore, proper controls are necessary. Moreover, when using the resazurin assay to determine dose-response curves for a compound, it can be difficult to infer from this assay alone whether a compound is mainly cytotoxic or cytostatic. Therefore, other methods, such as Annexin-PI apoptosis assays or clonogenic survival assays should be used to evaluate cell death and long-term survival.

3.2.2 Clonogenic survival assay

The capacity of a single cell to proliferate and form a visible colony of at least 50 cells can be evaluated in the clonogenic assay[269,270]. This cell survival assay has been used extensively in cancer research since the ability to form clones is a trait of cancer cells. Additionally, clonogenic assays can be applied to study stem cell biology[270,271]. A typical colony formation experiment includes seeding of adherent cells at very low densities (usually a few hundred cells per well) either before or after a treatment to equally distribute single cells on the surface. Thereafter, cells are allowed to grow until visible colonies are formed, which usually takes a few weeks, stained with crystal violet or methylene blue and finally counted[270].

The clonogenic assay is the preferred method when studying reproductive viability of cells after treatment with ionizing radiation[270]. However, it can also be applied to evaluate the effect of different gene manipulations (e.g., gene knockdowns and knockouts) and cytotoxic drugs on overall cell survival[270]. In **Paper I**, long-term survival of U2OS osteosarcoma cells was studied following siRNA-mediated depletion of MTHFD2 and overexpression of different siRNA-resistant MTHFD2 constructs, including the wildtype and a catalytically dead enzyme. In **Paper III**, cancer cell survival was determined after ionizing radiation and knockdown of MTHFD1 or MTHFD2 using the clonogenic assay in order to evaluate the importance of these enzymes for overall survival following induction of DNA damage.

Compared to short-term cytotoxicity assays which rely on cellular metabolic activity as a marker for viability, like the resazurin assay, the clonogenic assay has the advantage that it can provide information about the long-term cell reproductive capacity. It also enables experimental designs that include drug washouts to study whether a drug is cytotoxic or cytostatic. However, clonogenic assays are relatively time-consuming and they can be difficult to adapt for high-throughput screening, although recent efforts have been directed to coupling these assays to high-throughput screens[272]. It can also

be difficult to achieve a homogenous cell suspension with very few cells before seeding, which can lead to large variations between technical replicates. Moreover, not all cells form distinct colonies that are easily visualized, this depends largely on the specific characteristics of the cell, such as morphology and adherence to the surface.

3.3 Isolation of proteins on nascent DNA (iPOND)

To study the association of MTHFD1 and MTHFD2 with active replication forks, iPOND was performed in **Paper III**. This methodology includes pulsing cells with the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU) which becomes incorporated into newly synthesized DNA instead of thymidine. Since EdU contains an alkyne group, this allows a copper-catalyzed click chemistry reaction to covalently conjugate biotin-azide to the EdU-labelled DNA fragment[273]. Prior to biotin conjugation, protein-DNA complexes are cross-linked with formaldehyde to enable isolation of proteins that associate directly or indirectly with nascent DNA at replication forks. To purify the biotin-labelled complexes, cells are lysed and sonicated to solubilize the protein-DNA complexes, which are then captured using streptavidin-agarose beads. Protein-DNA crosslinks are reversed and captured proteins are eluted by boiling in SDS sample buffer, followed by Western blotting or mass spectrometry analysis[274–276].

In addition to identifying new replisome proteins, iPOND can also be applied to monitor chromatin maturation and to study the recruitment of DDR proteins to stalled forks[274–276]. For instance, altering the experimental design to include a chase period after the EdU pulse where cells are treated with an agent that blocks replication progression, like hydroxyurea, allows studies of protein dynamics upon replication stress.

Regardless of the type of iPOND experiment, proper controls should always be included in the experimental design to facilitate data interpretation[274–276]. A negative control, where either the biotin-azide is omitted from the click reaction mixture ("no click" sample) or cells are not pulsed with EdU, is necessary for all iPOND experiments to control for the specificity of the purification process, and helps to detect any potential problems, such as protein precipitation or proteins interacting non-specifically with the streptavidin beads. No protein-DNA complexes should be present in this sample after purification[274–276]. Moreover, it is also important to include a control immunoblot and probe for known replication proteins, like the proliferating cell nuclear antigen (PCNA), in order to validate successful purification of active replication forks[274–276]. Lastly, to distinguish between true replisome factors and proteins that associate with chromatin irrespective of active replication, it is crucial to have a thymidine chase control. In this sample, cells are incubated with thymidine after the EdU pulse prior to fixation and harvesting. Proteins that associate and travel with active replication forks are only enriched in the sample where thymidine chase was not included and they should therefore not be present in the chase sample[274–276].

Today, iPOND has an important place among the methods used to study replication fork biology[277]. Compared to conventional immunofluorescence imaging, it offers increased sensitivity and allows isolation of replisome proteins present at low abundance[275,276]. Moreover, iPOND applications that include pulse-chase experiments enable spatial and temporal analysis of protein dynamics at replication forks[274–276]. This method can also be combined with unbiased techniques for protein identification, such as MS, thus allowing identification of novel replisome proteins[278]. Additionally, MS-coupled iPOND can be combined with stable isotope labelling of amino acids in cell culture (SILAC), which permits a direct comparison of the protein abundances between two samples[279].

Although iPOND offers many advantages, it is a relatively labor-intensive method and since it lacks an amplification step, a large number of cells per sample (100 million per sample) are required to capture enough protein for detection when working with unsynchronized cells[275,277]. The large amount of starting material necessary and the subsequent need for a lot of incubator space were major drawbacks experienced first-hand when collecting data for **Paper III**. Moreover, performing iPOND is not trivial and requires technical skills to optimize the experiment. For instance, boiling samples in SDS sample buffer can sometimes also release proteins that are bound to the streptavidin beads in a non-specific manner. Using milder elution conditions, such as photocleaving the biotin-azide with the help of UV could be used as an alternative approach to release the protein-DNA complexes from the beads[275]. Additionally, since iPOND is an ensemble method, it can only offer a description of an average replication fork dynamics and therefore it does not take into account the heterogeneity between cells[275,277].

3.4 Immunofluorescence imaging of DNA damage and repair proteins

Upon DNA damage, DDR proteins are modified and accumulate at the sites of DNA damage to form distinct nuclear puncta, called damage or repair foci[280]. For instance, H2AX becomes phosphorylated at serine 139 following DSB induction (modification at the damage site)[234,281,282], whereas the HR protein RAD51 is recruited to the site of DNA damage (accumulation at the damage site)[282,283]. Repair foci can easily be detected in fixed or live samples using immunostaining and microscopy. In this thesis, fixed cells were first permeabilized, then stained with primary antibodies targeting the proteins of interest, followed by incubation with fluorophore-conjugated secondary antibodies and DNA counterstaining with DAPI. The fixation methods for each protein target were optimized empirically. To better visualize RPA32, RPA70 and 5-bromo-2'-deoxyuridine (BrdU)-labelled ssDNA foci (**Paper III**), cytoskeletal and loosely held nuclear proteins were removed *in situ* prior to fixation using the cytoskeleton (CSK) buffer[284].

Image-based detection of DNA repair factors holds several advantages against intensity-based methods, such as flow cytometry and Western blotting. These include, for instance, detection of changes in the subcellular localization of the protein of

interest, analysis of several parameters from a single image (e.g., number of foci per cell, foci size, fraction of foci-positive cells, fluorescence intensity of each individual focus), as well as the possibility to simultaneously stain for several DDR proteins, allowing studies of protein co-localization and repair pathway complexity[285]. In this thesis, confocal microscopy was chosen as the imaging technique instead of the conventional widefield microscopy to achieve better resolution and image quality[286].

Despite its many advantages, immunofluorescence microscopy can be time-consuming, especially if images are acquired manually as was done in this thesis. Moreover, it requires target-specific antibodies or tagging the protein of interest with a fluorescent fusion tag, such as the green fluorescent protein (GFP). In immunofluorescence imaging, lack of antibody specificity and unspecific staining constitute a major concern, and therefore new antibodies should always be validated for use in immunofluorescence. To verify specific staining with the primary antibodies targeting MTHFD1 and MTHFD2 used in **Paper III**, siRNA knockdown of the target proteins was performed.

In **Paper III**, the immunofluorescence microscopy studies focused on investigating the changes in subcellular localization of MTHFD2 following IR-induced DNA damage, as well as the effect of MTHFD2 depletion on the formation of nuclear repair foci. Both fluorescence intensity per nucleus or focus and the number of foci per nucleus were analyzed using CellProfiler[287] to allow automation of image analysis. Moreover, to complement image-based studies, subcellular fractionation and Western blotting were performed to examine the role of MTHFD2 in DNA repair.

3.5 Ethical considerations

The majority of the experiments presented in this thesis project included working with established human cancer, as well as non-transformed cell lines. Conduction of these *in vitro* experiments did not require any ethical permit. *In vivo* mouse xenograft studies described in **Paper I** were performed in accordance with the ethical guidelines by trained staff members, and approved by Stockholm ethical committee (ethical permits N217/15 and N89/14). For **Paper II** and **Paper III**, no ethical permits were required since experiments reported in these studies were done using only established human cancer cell lines and purified protein material.

4 Results

4.1 Paper I

Pharmacological targeting of MTHFD2 suppresses acute myeloid leukemia by inducing thymidine depletion and replication stress

The 1C folate metabolism enzyme MTHFD2 is normally expressed during early embryogenesis, but becomes reactivated and consistently overexpressed in tumors, making it an attractive anticancer target. While few inhibitors of MTHFD2 have previously been described, such as LY345899 and DS18561882, these compounds exhibit potency in the micromolar range and may not be optimal for clinical development. In this study, we sought to develop potent, low-nanomolar small-molecule inhibitors targeting MTHFD2, characterize the mechanism of action of these inhibitors in cancer cells and evaluate their therapeutic potential.

First, we validated knockdown of MTHFD2 with siRNA to impair proliferation of cancer cells and demonstrated that addition of thymidine was sufficient to rescue viability. Furthermore, we demonstrated that expression of RNAi-resistant wild-type but not catalytically dead MTHFD2 rescued viability, suggesting that enzymatic inhibition of MTHFD2 would recapitulate the effect observed with siRNA.

To find compounds suitable for hit expansion, we initially employed three high-throughput screening campaigns using biochemical *in vitro* assays, none of which yielded any suitable hits. Nevertheless, through a substrate-guided rational drug design and lead optimization approach, we managed to identify a novel series of small-molecule inhibitors, including TH7299, TH9028 and TH9619, which showed nanomolar affinity toward MTHFD2 in biochemical assays and improved cell activity as compared to LY345899. These inhibitors demonstrated antiproliferative efficacy *in vitro* cell assays, especially in AML and T-ALL Jurkat cells. They also displayed high selectivity towards cancer cells over nontumorigenic lymphoblastoid cells in viability and apoptosis assays. In CETSA assays, TH9028 and TH9619 were also more potent to thermally stabilize MTHFD2 in AML than non-tumorigenic B cells. Moreover, both TH9028 and TH9619 showed intratumor target engagement of MTHFD2 and prolonged survival in a mouse xenograft model of AML.

Co-crystal structures confirmed the binding of TH7299, TH9028 and TH9619 in the THF pocket of human MTHFD2. Using DARTS and CETSA, we confirmed that our inhibitors stabilized MTHFD2 against protease and thermal degradation, respectively, indicating intracellular target engagement. Although these inhibitors showed good selectivity toward MTHFD2 over a subset of other THF-dependent enzymes, including SHMT1, SHMT2, DHFR and TYMS, they still potently targeted the isozymes MTHFD1 and MTHFD2L

in biochemical activity assays. To address the relative contribution of MTHFD1 and MTHFD2 inhibition by our compounds to the observed antiproliferative effect in cancer cells, we tested TH9619 in *MTHFD2* CRISPR-Cas9 knockout cells and found increased resistance to the compound as compared to the wildtype cells. By the time of the study, we interpreted these results as evidence that our inhibitor series mainly exert their cytotoxic activity through direct inhibition of MTHFD2, a notion that we revisited and revised later in **Paper II**.

Mechanistically, our inhibitors caused depletion of thymidylate and subsequent uracil misincorporation into DNA, followed by induction of replication stress, DNA damage and apoptosis in cancer cells. In line with this model for mechanism of action through induction of replication stress, we also found that TH7299 and TH9619 synergized with DDR inhibitors, including ATR, CHK1 and WEE1 inhibitors.

In conclusion, this study showcases a new series of small-molecule inhibitors targeting the dehydrogenase/cyclohydrolase (DC) activity of MTHFD2, as well as MTHFD1 and MTHFD2L, and highlights their therapeutic potential in cancer treatment.

4.2 Paper II

Formate overflow drives toxic folate trapping in MTHFD1 inhibited cancer cells

In **Paper I**, we showed that loss of MTHFD2 resulted in increased resistance to TH9619, indicating MTHFD2 as a drug target. However, we failed to metabolically rescue the antiproliferative effect of TH9619 on cancer cells with the purine derivative hypoxanthine. Instead, addition of thymidine rescued the cells, which was unexpected considering that thymidylate synthesis can be sustained independently of the MTHFD enzymes by the cytosolic SHMT1[101]. Therefore, in this second study we aimed to further examine the mechanism of action of our MTHFD1/2 inhibitors, especially TH9619, and clarify whether these inhibitors directly target MTHFD1 and/or MTHFD2.

Using a combination of methods, including metabolic rescue experiments, determination of formate release rates and CETSA, we found that TH9619 and related compounds targeted nuclear but not mitochondrial MTHFD2, thus allowing the overflow of formate from the mitochondria to continue. We then investigated, whether inhibition of the DC domain of MTHFD1 (MTHFD1(DC)) occurring downstream of mitochondrial formate release contributed to the toxicity of our MTHFD1/2i. To this end, we introduced a TH9619-resistant mutant of MTHFD1(DC) into SW620 colorectal cancer cells using CRISPR-Select and observed increased cell proliferation, indicating partial resistance to TH9619 in the mutant cells. Moreover, we could show stabilization of MTHFD1 by TH9619 in DARTS assays. We also found in our metabolic tracing experiments that *MTHFD2*-/- cells lost their capacity to synthesize purines *de novo* as opposed to the wildtype cells when treated with TH9619 or TH9975, which is another MTHFD1/2i from the same

compound series. Collectively, these results provided evidence for the notion that the toxicity of our MTHFD1/2 inhibitors is mainly mediated by inhibition of the cytosolic MTHFD1(DC), and not by directly targeting mitochondrial MTHFD2.

We next identified significant differences in sensitivity to TH9619 treatment in regular versus dialyzed (purine-depleted) serum between MTHFD2-expressing and *MTHFD2*-null cells using metabolic rescue experiments. When cultured in media supplemented with dialyzed serum, *MTHFD2* wildtype cells lost their sensitivity to TH9619 and TH9975, whereas *MTHFD2-/-* cells became more sensitive, indicating different modes of TH9619-induced toxicity in cells with intact versus deficient mitochondrial 1C metabolism. Addition of hypoxanthine restored the sensitivity of wildtype cells to TH9619 and TH9975, which could be reversed with simultaneous thymidine supplementation. In contrast, cell viability was rescued by hypoxanthine and sodium formate in *MTHFD2*-null cells, while thymidine had no effect. Thus, we concluded that the toxicity of our MTHFD1/2i was mainly due to thymidylate depletion in *MTHFD2* wildtype cells and purine deficiency in *MTHFD2-/-* cells. Moreover, later analysis of cell proliferation and apoptosis in these cells revealed that only TH9619-induced thymidylate depletion led to significant cell death, whereas purine loss was cytostatic.

Since hypoxanthine seemed to modulate the toxicity of our MTHFD1/2i, we postulated that hypoxanthine would inhibit *de novo* purine synthesis, and that simultaneous inhibition of MTHFD1(DC) in cells with intact mitochondrial formate production would ultimately cause decreased flux of the folate intermediate 10-CHO-THF into the *de novo* purine synthesis and thymidylate synthesis pathways. Trapping of free THF as 10-CHO-THF could also prevent SHMT1 from supporting thymidylate synthesis. To directly test this hypothesis, we quantified cellular 10-CHO-THF, and observed an accumulation of 10-CHO-THF upon TH9619 treatment in SW620 *MTHFD2* wildtype cells, but not in *MTHFD2-/-* cells, and this became even more pronounced in the presence of hypoxanthine. We then created a comparable toxic 10-CHO-THF trap in *MTHFD2-/-* cells, which are normally unable to synthesize mitochondrial formate, by simultaneously combining TH9619, hypoxanthine and sodium formate. This resulted in TH9619 toxicity due to thymidylate deficiency and a subsequent increase in apoptotic cells, thus verifying the importance of mitochondrial overflow to the proposed folate trap mechanism. Furthermore, we could release the 10-CHO-THF trap and rescue the viability of SW620 *MTHFD2* wildtype cells by supplementation of 5-amino-4-imidazolecarboxamide ribonucleoside (AICAr), which, once inside the cell, is subsequently converted to FAICAR, freeing THF from 10-CHO-THF in the process.

In summary, this paper details a previously uncharacterized 10-CHO-THF trapping mechanism upon inhibition of MTHFD1(DC) in MTHFD2-expressing cancer cells, which causes thymidylate depletion and cell death. We propose that TH9619 and related

compounds acting through this mechanism could potentially be used therapeutically to kill cancer cells.

4.3 Paper III

Targeting MTHFD2 impairs homologous recombination and sensitizes cancer cells to PARP inhibitors

While the mitochondrial role of MTHFD2 is well characterized, its nuclear functions remain more elusive. In **Paper III**, we set out to study the involvement of MTHFD2 in DNA replication and repair in cancer cells. Previous immunofluorescence studies suggest that MTHFD2 co-localizes with active replication forks[98]. To validate this, we employed the iPOND assay and could show that the association of MTHFD2 was not only restricted to nascent replication forks. Interestingly, we could detect a modest increase in chromatin-bound MTHFD2 following etoposide treatment, as well as a significant elevation of MTHFD2 protein levels, particularly in the nucleus after prolonged treatment with hydroxyurea, suggesting that MTHFD2 could be involved in the DNA damage response at stalled/collapsed replication forks.

Next, we investigated the subcellular localization of MTHFD2 following IR treatment and observed a rapid accumulation of MTHFD2 in the nucleus. Moreover, MTHFD2 co-localized with γH2AX after IR, supporting the notion that MTHFD2 is recruited to sites of DNA damage. We also found significantly reduced clonogenic survival in U2OS and HCT116 cells upon MTHFD2 siRNA depletion and IR. In addition, CRISPR-Cas9-generated *MTHFD2*$^{-/-}$ SW620 cells showed a significant reduction in proliferation following IR treatment as compared to the more radioresistant wildtype cells. Interestingly, when examining the isoenzyme MTHFD1 we could not detect recruitment of this enzyme to the nucleus following IR. In addition, depleting MTHFD1 with siRNA yielded inconsistent results between U2OS and HCT116 cells in clonogenic assays after IR treatment. In conclusion, these data suggest that MTHFD2 may play a more prominent role in the repair of IR-induced DNA damage than MTHFD1.

To dissect the repair pathways that MTHFD2 could participate in, we inhibited the three main DDR kinases ATM, ATR and DNA-PK and saw that MTHFD2 nuclear recruitment upon IR was abolished in ATM- and DNA-PK-inhibited cells, suggesting a potential role of MTHFD2 in ATM- and DNA-PK-mediated DSB repair. We then evaluated the effect of MTHFD2 depletion on the generation of ssDNA and DNA end resection by visualizing BrdU, RPA70 and RPA32 foci. Silencing of MTHFD2 with siRNA led to significantly less BrdU foci, as well as impaired focal accumulation of both RPA70 and RPA32 following IR, suggesting that MTHFD2 is necessary for DNA end resection. We also observed impaired BRCA1 phosphorylation, which further indicates a role of MTHFD2 in the early steps of HR to facilitate proper end resection. Moreover, knocking down MTHFD2 resulted in decreased nuclear recruitment of the key HR factor RAD51 and a subsequent reduction

in HR activity. We also observed impaired NHEJ activity upon MTHFD2 knockdown, suggesting a role of MTHFD2 in mediating both HR and NHEJ.

We also hypothesized that targeting MTHFD2 could conditionally generate a HR defect and sensitize HR-proficient cancer cells to PARP inhibitors. In line with this, we observed that MTHFD2 knockdown sensitized cancer cells to talazoparib and pharmacological inhibition of MTHFD2 synergized with olaparib to induce apoptosis especially in cancer cells, while largely sparing non-transformed cells. Moreover, TH9619 synergized with talazoparib in THP-1 and SW620 cells.

In conclusion, this study identifies a key role of MTHFD2 in the early steps of DSB repair to facilitate DNA end resection and highlights the interdependency between MTHFD2 expression and repair proficiency in cancer which could potentially be exploited therapeutically.

5 Discussion and future perspectives

5.1 Therapeutic potential of MTHFD1/2 inhibitors in cancer

Targeting the 1C metabolism with antifolate drugs like methotrexate and pemetrexed has long been employed in the clinic for the treatment of hematological and solid tumors[62,64–66]. However, these drugs exhibit multi-target activity (polypharmacology) and diverse mechanisms of action which can cause toxicities in healthy tissue. Moreover, tumors can develop resistance, resulting in relapse[62,288]. Thus, new therapeutic approaches are needed. In this thesis, we aimed to address the need for new and more selective cancer therapies by specifically targeting the MTHFD2-dependent 1C folate pathway and developed a series of potent, small-molecule MTHFD1/2 inhibitors, including the lead candidate TH9619. The hypothesis was that we would be able to inhibit the 1C pathway while causing less adverse effects compared to the classical antifolates.

In **Papers I** and **II**, we validate MTHFD2 as a cancer target and detail the mechanism of action of the MTHFD1/2 inhibitors. We show that these compounds mainly caused cytotoxicity in MTHFD2-expressing cancer cells by suppressing *de novo* thymidylate production and inducing misincorporation of uracil into DNA, which leads to increased replication stress. At first, we proposed that inhibition of MTHFD2 (mitochondrial and nuclear) is required for the observed antiproliferative effect of these compounds based on our CRISPR-Cas9 target validation data (**Paper I**). However, a more detailed analysis later revealed that TH9619 did not disrupt production of mitochondrial formate and failed to stabilize mitochondrial MTHFD2 in CETSA assays, indicating that TH9619 does not inhibit mitochondrial MTHFD2 (**Paper II**). Instead, we concluded that TH9619 mainly kills cancer cells by inhibiting MTHFD1 (DC). Moreover, we showed that TH9619 also engages with nuclear MTHFD2 and might therefore interfere with the nuclear functions of MTHFD2, affecting processes like DSB repair (**Paper III**).

Importantly, our findings in **Paper II** highlight the importance of intact mitochondrial 1C metabolism for the toxicity of TH9619. Upon TH9619 treatment, overflow of formate from the mitochondria continues and this formate is converted to 10-CHO-THF by MTHFD1 (FS). Since the DC domain of MTHFD1 is inhibited by TH9619, 10-CHO-THF cannot be converted further into 5,10-CH_2-THF, which impairs *de novo* thymidylate synthesis. We show that TH9619 induced accumulation of 10-CHO-THF in SW620 (**Paper II**) and MDA-MB468 cells (data not shown), and this was further enhanced by physiological levels of the purine derivative hypoxanthine which indirectly inhibits consumption of 10-CHO-THF for the *de novo* purine synthesis. Ultimately, cells died of thymidylate depletion, and addition of external thymidine was sufficient to restore cell viability and decrease apoptosis. Interestingly, this trapping mechanism did not seem to occur in methotrexate-treated cells, suggesting that accumulation of 10-CHO-THF is unique for

TH9619 and related compounds. We observed no significant accumulation of 10-CHO-THF ('folate trap') in *MTHFD2*$^{-/-}$ cells treated with TH9619, indicating that MTHFD2-driven formate overflow is essential for TH9619-induced thymidylate depletion and cell death. Based on our findings, we propose that accumulation of 10-CHO-THF upon MTHFD1 (DC) inhibition in MTHFD2-expressing cells depletes the cytosolic pool of free THF and starves SHMT1 from its substrate. Thus, generation of 5,10-CH_2-THF is blocked from both directions, impairing the *de novo* thymidylate synthesis. However, studying the changes in THF levels upon treatment with TH9619 remains a topic for future studies.

Given the fact that MTHFD1 (DC) is the main target of TH9619, our initial results in **Paper I** showing that MTHFD2, but not MTHFD1 CRISPR-Cas9 knockout cells displayed increased resistance to the compound seem puzzling. However, we still detected faint bands for MTHFD1 protein on our Western blots, indicating knockdown of MTHFD1 rather than knockout. Thus, the direct target of TH9619 inhibition was marginally expressed in these cells. In addition, MTHFD2 was still expressed in these cells, allowing formate overflow and thus generating the toxic 10-CHO-THF trap. Additionally, *MTHFD2*$^{-/-}$ cells showed resistance to TH9619 in **Paper I** since no folate trap could be generated in these cells. Moreover, these cells were cultured in media containing regular FBS (which also contains hypoxanthine), which rescues the cytostatic effect of TH9619 due to purine depletion.

Additionally, considering our current understanding of the 1C pathways, it might seem counterintuitive that external thymidine, but not hypoxanthine could restore viability of U2OS cells upon depletion of MTHFD2 with siRNA (**Paper I**). It would be expected that knockdown of MTHFD2 would have an effect on both purine and thymidylate synthesis, or alternatively would not have any considerable effect at all if MTHFD2 silencing is compensated by the reversal of the 1C cycle and activity of SHMT1 in the cytosol[101]. However, these experiments were performed in cell culture media containing regular FBS, which might have rescued the effect on purine synthesis. Our preliminary data also suggests that U2OS cells express relatively little SHMT1 (data not shown), which might partly explain why they cannot compensate for the lack of 5,10-CH_2-THF and suffer from thymidylate depletion, which can then be rescued by addition of thymidine.

The mechanism of action of TH9619 has also therapeutic implications and helps to explain the cancer-specificity of this compound observed so far. In highly proliferative cells, like cancer cells, the mitochondrial 1C metabolism becomes upregulated and is required to generate formate overflow, which is a hallmark of cancer[289–292]. MTHFD2-driven, excessive release of formate from the mitochondria is essential for the generation of 10-CHO-THF trap and depletion of free THF upon inhibition of MTHFD1 (DC) by TH9619. In **Paper II**, we show that removal of MTHFD2 abolishes mitochondrial formate release, as well as accumulation of 10-CHO-THF following treatment with TH9619 and confers resistance to the inhibitor in the presence of physiological levels of hypoxanthine. We propose that this resembles healthy, non-proliferating cells that

normally express low levels of MTHFD2 protein[47]. In the future, it would be interesting to study formate release and levels of 10-CHO-THF upon TH9619 treatment in non-malignant cells to further validate this.

In addition to the mechanism of action of TH9619, we argue that the cancer-specificity of this compound is also partly due to enhanced target engagement in cancer cells compared to non-transformed cells. In **Paper I**, we show that TH9619 displayed increased thermal stabilization of MTHFD2 in HL-60 leukemia cells compared to non-malignant LCL-889 lymphoblastoid cells. This is likely due to the enhanced uptake of folates (and other folate-like compounds) to support biomass generation in cancer cells[74]. While we only addressed the differences in target engagement with MTHFD2 by TH9619 between cancer and non-malignant cells, we envision that a similar trend may hold true also for MTHFD1.

In our studies, TH9619 appeared less promiscuous compared to classical antifolates, such as methotrexate or pemetrexed. In **Paper I**, we show that TH9619 did not stabilize DHFR, TYMS, SHMT1, SHMT2 or MTHFD1L in DARTS assays, suggesting that it is unlikely to bind these enzymes. Moreover, data from our biochemical activity assays indicated that TH9619 did not drastically inhibit the enzymatic activity of DHFR, TYMS, SHMT1 or SHMT2. In the future, it would be interesting to expand these off-target studies and investigate the effect of TH9619 in the THF-dependent *de novo* purine synthesis enzymes GART and ATIC. Given that TH9619 cannot enter the mitochondria, it is also unlikely to inhibit MTHFD2L, which may limit toxicities in healthy tissues expressing this enzyme. Interestingly, MTHFD1/2 inhibitors exclusively reduced replication fork speed in leukemia cells, while methotrexate significantly slowed down replication speed also in non-tumorigenic lymphoblastoid cells (**Paper I**).

Collectively, our findings suggest that MTHFD1/2 inhibitors may display higher tolerability compared to classical antifolates due to their specific mechanism of action and lack of polypharmacology. Nevertheless, in **Paper I**, we observed a decreased number of red and white blood cells in mice kept on low-folate diet and treated with TH9616, indicating potential toxicity in the bone marrow, which is characteristic of antifolate therapy. However, more comprehensive toxicology studies are required in the future to better elucidate potential toxicities and determine safe dosing of TH9619.

Lastly, combining other anticancer drugs with MTHFD1/2 inhibitors may also have translational implications and should be studied in more detail in the future. In **Paper I**, we found that MTHFD1/2 inhibitors were more selective in reducing replication speed in cancer cells compared to the ATR inhibitor VE-821, which also affected replication in non-tumorigenic cells. Moreover, we found strong synergy between MTHFD1/2 inhibitors and ATR and CHK1 inhibitors in cancer cells. It would be interesting to further examine, whether co-administration of TH9619 could help to broaden the therapeutic index of

these replication stress checkpoint inhibitors in animal models. Other combination strategies should also be explored in the future. For instance, given that MTHFD1/2 inhibitors show efficacy in colorectal cancer cell lines (**Paper II** and unpublished data), it would be worth investigating, whether these compounds can synergize with 5-FU, oxaliplatin or irinotecan which are used in for the treatment of colorectal cancer. Moreover, since MTHFD1/2 inhibitors induce DNA damage, it would be interesting to evaluate whether these compounds can synergize with cancer immunotherapy[293], such as monoclonal antibodies targeting PD-L1.

In summary, **Papers I** and **II** demonstrate a promising approach to kill cancer cells with MTHFD1/2 inhibitors. However, it remains to be evaluated whether these compounds can show efficacy and tolerability in several pre-clinical animal models and clinical trials, and whether they can ultimately be added to the arsenal of cancer therapies.

5.2 Role of MTHFD2 in DSB repair and its therapeutic implications

In **Paper III**, we demonstrate a nuclear function of MTHFD2 in regulating the early steps of DSB repair. Following IR treatment, MTHFD2 rapidly accumulated in the nuclear compartment, which was dependent on the activity of the ATM and DNA-PK kinases. We also observed increased chromatin association of MTHFD2 after IR and found that MTHFD2 co-localized with DNA damage sites. Moreover, we observed defective DNA end resection upon MTHFD2 knockdown, quantified as decreased RPA32, RPA70 and BrdU (marker for formation of ssDNA) foci formation following IR as compared to the control. MTHFD2 knockdown also resulted in decreased nuclear accumulation of RAD51 and significantly reduced HR activity following IR, which was expected since defective end resection impairs HR. Based on these findings, we therefore propose that MTHFD2 is required for proper end resection during HR repair. This is in line with a previous report that ascribed MTHFD2 to have a role in HR in mouse pluripotent stem cells[255]. This study suggested that MTHFD2 directly binds CDK1 and EXO1 to promote end resection. However, we could not confirm this in our experiments (data not shown), which could partly be due to the fact that we used cancer cell lines and not mouse stem cells in our studies.

Interestingly, both knockdown and knockout of MTHFD2 abolished phosphorylation of BRCA1 upon IR treatment. This is intriguing, since BRCA1 is known to be phosphorylated by ATM and promote initial end resection through its interaction with the MRN complex and CtIP during HR repair[223,240,294]. Besides HR, BRCA1 has also an accessory function in NHEJ[295,296]. Consistent with previous findings by Li et al.[100], which suggest a role of MTHFD2 also in NHEJ, we observed decreased NHEJ activity in MTHFD2-depleted cells following IR. Moreover, DNA-PK kinase, which plays an integral part in NHEJ, promoted nuclear accumulation of MTHFD2. Overall, our findings would suggest that MTHFD2 acts downstream of ATM and DNA-PK but upstream of BRCA1 activation and end resection.

However, it remains to be addressed how exactly MTHFD2 regulates DNA damage signaling and BRCA1 upon induction of DSBs. For instance, it would be interesting to study the nuclear interactome of MTHFD2 following IR treatment in order to establish a more comprehensive picture of the mechanisms behind MTHFD2-mediated regulation of NHEJ and HR repair. To this end, methods such as the improved proximity-dependent biotin identification (BioID2)[297,298] and co-immunoprecipitations could be utilized.

Our findings in **Paper III** regarding the ATM- and DNA-PK-mediated nuclear accumulation of MTHFD2 also raise the question of how MTHFD2 is translocating into the nucleus to co-localize with DNA damage sites. Using co-immunoprecipitations, we tested direct binding between MTHFD2 and ATM or DNA-PK but could not detect any direct interaction between the proteins, suggesting indirect regulation of MTHFD2 nuclear accumulation by ATM and DNA-PK. However, we did not investigate any putative phosphorylations or other post-translational modifications on MTHFD2 following IR. Thus, the exact mechanism behind nuclear translocation of MTHFD2 upon DNA damage remains a topic for future research. For example, studies investigating potential post-translational modifications on MTHFD2 following induction of DSBs could provide further insight. Interestingly, it has been reported previously that other 1C enzymes like MTHFD1 and SHMT1 undergo SUMOylation upon DNA damage, which allows their nuclear translocation[86–88,90]. Therefore, it would be interesting to study if this also applies to MTHFD2.

Another interesting question that remains to be addressed in future studies is whether the catalytic function of MTHFD2 and production of 5,10-CH_2-THF are required for its nuclear role in the DNA damage response following IR treatment. A previous report linking MTHFD2 to NHEJ would suggest a non-catalytic function[100]. On the contrary, a report identifying an additional nuclear role of MTHFD2 in regulating mitosis and centromere stability proposes that catalytic activity of MTHFD2 is needed[299]. When we supplemented cells with thymidine or sodium formate, we could not rescue the clonogenic survival of MTHFD2-depleted U2OS or HCT116 cells following IR treatment (data not shown). Moreover, addition of sodium formate to SW620 *MTHFD2*$^{-/-}$ cells did not restore their proliferation to a similar level with the wildtype cells (data not shown). These preliminary findings suggest that generation of thymidylate through the mitochondrial MTHFD2 activity is unlikely to be responsible for the effect of MTHFD2 on DSB repair. However, further studies are needed to fully elucidate the involvement of catalytic activity of MTHFD2 in DNA damage response. For instance, it would be interesting to overexpress siRNA-resistant MTHFD2 wildtype or catalytically dead mutant enzyme in cells and examine, whether the catalytically inactive mutant could reverse the negative effects of MTHFD2 knockdown on RPA foci formation or BRCA1 phosphorylation following IR. Moreover, short-term treatment of cells with the MTHFD1/2 inhibitor TH9619 could also be used to understand, whether inhibition of the DC activity

of MTHFD2 recapitulates the phenotype seen with knockdown/knockout of MTHFD2 following IR treatment.

Previously, MTHFD1 has also been shown to localize to the nucleus and bind chromatin[89,97]. Therefore, we wanted to study if it could regulate DSB repair following IR or whether MTHFD2 was the main isoenzyme involved in the IR response. We could not detect any increased nuclear recruitment of MTHFD1 upon IR treatment and found also no evidence of impaired BRCA1 phosphorylation in irradiated cells upon MTHFD1 knockdown. Moreover, depletion of MTHFD2 more consistently impaired clonogenic survival after IR treatment as compared to silencing of MTHFD1. Thus, we propose that MTHFD2 has a more prominent role in regulating DSB repair following IR treatment than MTHFD1. To strengthen this claim, it would be important to also show that depletion of MTHFD1 does not impair end resection to the same extent as knockdown of MTHFD2. To address this, the effect of MTHFD1 silencing on formation of RPA foci could be studied and compared to that of MTHFD2 knockdown.

To study the function of MTHFD1 and MTHFD2 in DSB repair, we mainly employed siRNA-mediated silencing throughout the entire **Paper III**. However, both isoenzymes contain a DC domain and share considerable sequence homology[82]. Therefore, the validity of the conclusions drawn from the results depends on the specificity of the siRNAs used. In theory, it would be possible that an siRNA designed to silence MTHFD2 could also target MTHFD1 (DC), making it hard to distinguish between MTHFD1- and MTHFD2-mediated effects. However, when validating successful knockdown on Western blot, we could not detect any significant cross-reactivity, indicating that our siRNAs used against MTHFD1 and MTHFD2 were specific enough.

The role of MTHFD2 in DSB repair identified in **Paper III** may also have therapeutic implications. Importantly, we observed that otherwise relatively radioresistant SW620 cells could be sensitized to IR treatment upon MTHFD2 knockout. Moreover, we found increased MTHFD2 mRNA levels especially in radioresistant ALL patients following IR when analyzing available gene expression arrays. These findings collectively suggest that targeting MTHFD2 may open up novel strategies to combat radioresistance and this should be further tested. Interestingly, we also showed that pre-treatment with the MTHFD1/2 inhibitor TH9619 resulted in increased levels of DNA damage in TC71 Ewing sarcoma cells following IR treatment. This suggests that pharmacological targeting of MTHFD2 can be used to modulate DSB repair activity in cancer cells and encourages testing IR in combination with MTHFD1/2 inhibitors in the future. It would also be interesting to examine whether TH9619 can alter the IR response similarly to MTHFD2 knockdown/knockout as observed in **Paper III**. For example, the effect of short-term treatment with TH9619 on end resection following IR could be studied. An interesting question is also whether TH9619, which is a small molecule, can disrupt any potential protein-protein interactions MTHFD2 might have in DSB repair. Since TH9619 is not an

MTHFD2-specific compound, distinguishing between inhibition of nuclear MTHFD2 and impaired thymidylate synthesis through targeting MTHFD1 (DC) may, however, be challenging when treating cells long-term with TH9619. It would be interesting to develop MTHFD1-specific compounds and compare them to TH9619.

In addition to its radiosensitizing potential, targeting MTHFD2 may also sensitize repair-proficient cancers to PARP inhibitors by generating an HR defect. In **Paper III**, we showed that TH9619 synergized with the PAPR inhibitor talazoparib *in vitro* in THP-1 leukemia cells and to a lesser extent in SW620 colorectal cancer cells. However, lack of *in vivo* models remains a limitation of this study. Therefore, it would be important to further assess this combination in relevant solid cancer cell models, as well as animal models to enable us to draw more definitive conclusions about the translational implications of MTHFD1/2 inhibitors in this context.

5.3 Challenges in clinical translation of MTHFD1/2 inhibitors

Overall, this thesis presents promising preclinical findings regarding the development of MTHFD1/2 inhibitors as potential anticancer agents. However, to translate these compounds into actual cancer therapies poses also challenges which need to be addressed in future studies.

First, standard mouse xenograft models are likely not suitable for predicting TH9619 efficacy in humans due to the striking differences in metabolite levels between the two species. In mouse plasma, the levels of thymidine and folate are significantly higher (at least 10-fold), whereas hypoxanthine levels are much lower (100-1,000-fold) than in human plasma[300–302]. As we demonstrate in **Papers I** and **II**, folate, thymidine and hypoxanthine levels greatly influenced the efficacy of TH9619. Keeping mice on low-folate diet is one way effectively reduce folate levels in these animals[302–304]. In **Paper I**, we show that feeding the mice with a low-folate diet improved the antitumor activity of TH9619 as compared to standard diet. However, low-folate diet did not decrease high plasma thymidine levels in mice. Conversion of thymidine to dTMP by TK1 and TK2 can effectively bypass inhibition of *de novo* thymidylate synthesis[54,58,305]. Previous studies on TYMS inhibitors, such as raltitrexed, have highlighted the problem with high thymidine levels in murine models, which complicates the study of anticancer effects of drugs targeting *de novo* thymidylate synthesis[300,306]. Therefore, means to combat high thymidine levels in mouse plasma are likely needed in the future when evaluating the antitumor efficacy of TH9619 in murine xenograft models. Currently, a limitation of the studies presented in this thesis is their lack of several preclinical *in vivo* models. One potential way forward could be the use of TK-deficient tumor models[304,307,308].

Second, metabolic plasticity might pose a challenge for translating TH9619 into a clinically relevant anticancer drug. Even though the level of thymidine in human plasma is relatively low, cancer cells can still shift to pyrimidine salvage which may reduce or

even abolish the efficacy of TH9619. Moreover, human plasma also contains uridine (roughly 3.6 μM)[301], which can be converted intracellularly into UMP by UCK and ultimately contributes to the generation of dTTP[54,58]. However, it remains to be tested whether pyrimidine salvage has a significant effect on the clinical outcome of TH9619 as monotherapy.

Another important question is whether TH9619 can still show efficacy in tumors treated with other antifolates, like methotrexate and pemetrexed, or antimetabolites, such as 5-FU. This is relevant since patients will have received chemotherapy and may have likely developed resistance before trying TH9619. Pemetrexed is used to treat for example non-small cell lung cancer[309], while 5-FU is often used for the treatment of colorectal cancer, for instance[310]. The 10-CHO-THF trapping mechanism of TH9619 (**Paper II**) differs from the mechanism of action of other antifolates (methotrexate) and antimetabolites targeting TYMS (5-FU), however, they all ultimately disrupt *de novo* thymidylate synthesis and will likely share at least some common resistance mechanisms. Therefore, it would be interesting to test *in vitro* whether TH9619 can still have a cytotoxic effect on cancer cell lines that have acquired resistance to methotrexate, pemetrexed or 5-FU in order to elucidate the extent of cross-resistance.

Lastly, predicting patient groups that are sensitive to TH9619 treatment may be challenging. In **Paper I**, we found that not all leukemia cell lines responded to TH9619. Preliminary results from our group and collaborators also show that a substantial number of hematological and solid tumor cell lines do not respond to TH9619 (data not shown). In **Paper II**, we propose that MTHFD2 overexpression and formate overflow are important factors contributing the cytotoxic effect of TH9619 via trapping of 10-CHO-THF. However, these likely represent only a fraction of the predictors for TH9619 sensitivity, and the reality is probably much more complex than what we have uncovered so far. Folate trap may also not be efficient enough in some cancer cells that do not respond to TH9619, which would allow SHMT1 to generate 5,10-CH_2-THF for thymidylate synthesis. So far, we have only tested the accumulation of 10-CHO-THF upon TH9619 treatment in a few responsive cell lines. Predicting responses to TH9619 will potentially require establishing regression models with multiple predictors. For instance, establishing TH9619-resistant cancer cell lines would not only provide important information on the resistance mechanisms, but also generate important data which could be built into the models predicting TH9619 response. It would be interesting to establish these drug resistant cancer cell lines in the future and compare their mRNA and protein expression to parental cells. In the future, collaborative research will be needed to address additional factors contributing to TH9619 sensitivity and identify potentially responsive patient groups.

5.4 Concluding remarks

The constituent papers of this thesis showcase the development of MTHFD1/2 inhibitors, including the lead candidate TH9619, elucidate their complex mechanism of action and discuss their therapeutic potential in cancer. Moreover, our findings shed light on the nuclear role of MTHFD2 in DNA repair. Future research should be devoted to evaluating the efficacy of MTHFD1/2 inhibitors in various cancer types, investigating resistance mechanisms and combination strategies with other therapies, identifying potentially responsive patient groups and exploring the detailed molecular mechanisms behind MTHFD2-regulated DSB repair.

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